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The Journal of Biochemistry, Vol. 43, No. 4, 1956

STUDIES ON CONJUGATION OF S^{35}-SULFATE WITH PHENOLIC COMPOUNDS

III. METABOLISM OF *p*-CRESOL, *p*-HYDROXYBENZALDEHYDE AND *p*-HYDROXYBENZOIC ACID IN RAT LIVER*

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(Received for publication, March 10, 1956)

The *in vivo* experiments on the fate of *p*-cresol, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid have been studied by various workers. They have been reviewed by Williams in the Detoxication Mechanisms (*1*). Since then, several studies have been made *in vivo* by Bray, Thorpe *et al.* (*2–3*) and Hartles, Williams (*4*). However there has been no report about *in vitro* experiment, except the authors' previous report with *p*-cresol that it is conjugated with S^{35} -sulfate in the presence of rat liver slices (*3*). In the present study the metabolic pathways of the above substances were studied by the aid of S^{35} -sulfate which conjugates with them and their metabolites. Paper chromatography and radioautography have been used to examine the conjugates.

EXPERIMENTALS AND RESULTS

The livers were excised immediately from white rats after they were killed and kept cold in an ice cold Krebs' Ringer-phosphate solution of which magnesium sulfate was substituted by magnesium chloride so as to exclude sulfate.

Further experiments were conducted at the condition already

An account of the work described in this paper was presented at the 75th meeting of the Japanese Biochemical Society in Kanto District at the University of Tokyo on 12 March 1955.

described in the authors' previous reports (*5*, *6*). Ascending chromatography on Whatman No. 1 paper or Toyo No. 50 paper was used, and the developing solvent employed was *n*-butanol-acetic acid-water (4 :1 :1). The chromatograms were developed for about 16 hours. The sulfate conjugates of *p*-cresol and *p*-hydroxybenzoic acid were synthesized by

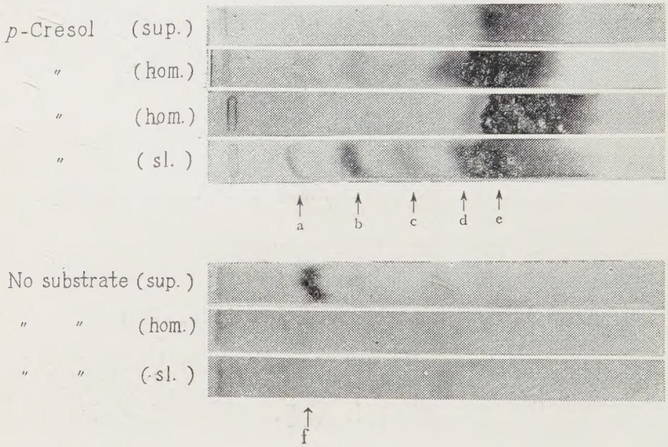


FIG. 1. Sulfate conjugates of *p*-cresol with rat liver.

Incubation with the supernatant: Supernatant (50%) 0.1 ml.+K ATP 1.5 mg.+20 μg of the substrate+S^{35}-sulfate, shaken in air phase at 37.5° for 1 hr. Incubation with the homogenate: Homogenate (50%) with KCl-Ringer-phosphate-succinate 0.1 ml.+KATP 0.5 mg. +20 μg. of the substrate+S^{35}-sulfate, shaken in O_2 at 37.5° for 1 hr.

Incubation with liver slices: Liver slices 0.1 g.+Ringer-phosphate-succinate 0.1 ml.+20 μg. of the substrate+S^{35} -sulfate, shaken in O^2 at 37.5° for 1 hr.

After the deproteinazation with trichloracetic acid, inorganic S^{35}-sulfate was removed with $BaCl_2$ and nonisotopic SO_4.

Paper chromatography was conducted by the solvent of CH_3-COOH, H_2O, *n*-butanol (1:1:4) for 16 hours.

a. Sulfate conjugate of *p*-hydroxybenzoic acid derivative (probably *p*-hydroxyhippuric acid)
b. Sulfate conjugate of *p*-hydroxybenzaldehyde
c. Sulfateconjugate of *p*-hydroxybenzoic acid
d. Sulfate conjugate of *p*-hydroxybenzyl alcohol (probably)
e. Sulfate conjugate of *p*-cresol
f. Byproduct of the intermediate for sulfate conjugation with the supernatant

chlorosulfonic acid in the medium of pyridine plus chloroform. (*7*).

Results with p-Hydroxybenzoic Acid—*p*-Hydroxybenzoic acid gave a S^{35}-sulfate conjugate with the liver supernatant, its Rf value being 0.31. The sulfate conjugate of *p*-hydroxybenzoic acid made by the reaction with chlorsulfonic acid in the medium of chloroform plus pyridine also gave a spot at Rf 0.31 which was visualized by the retarded color reaction with 2,6-dibromoquinone-4-chloroimide after hydrolysis with hydrochloric acid. From this result the substance which showed the spot of S^{35}-sulfate at the Rf value of 0.31 may be concluded to be the sulfate conjugate of *p*-hydroxybenzoic acid. But in the precence of liver slices,

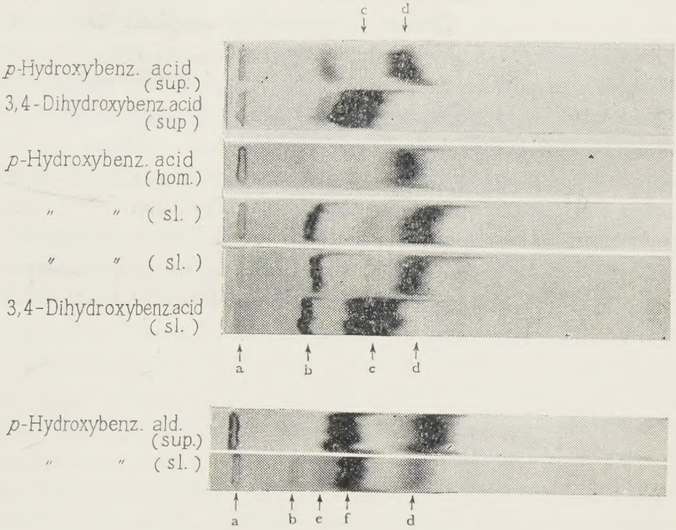


FIG. 2. Sulfate conjugates of *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde and 3,4-dihydroxybenzoic acid with rat liver.

Conditions were the same as Fig. 1.

a. Inorganic sulfate
b. Sulfate conjugate of *p*-hydroxybenzoic acid or 3,4-dihydroxybenzoic acid derivatives (probably *p*-hydroxyhippuric acid or 3,4-dihydroxyhippuric acid)
c. Sulfate conjugate of 3,4-dihydroxybenzoic acid
d. Sulfate conjugate of *p*-hydroxybenzoic acid
e. Sulfate conjugate of 3,4-dihydroxybenzaldehyde
f. Sulfate conjugate of *p*-hydroxybenzaldehyde

there appeared three spots, namely at the Rf value of 0.31, common to that with the liver supernatant, and two others. The one at 0.22 showed faint radioautogram and may be identified from its Rf value as the S^{35}-sulfate conjugate of 3,4-dihydroxybenzoic acid. No further experiment was conducted but another substance at Rf 0.11 seems to be the sulfate conjugate of a metabolite of *p*-hydroxybenzoic acid, probably modified at the carboxyl group by glycine conjugation.

Results with p-Hydroxybenzaldehyde—Experiments with the liver supernatant showed the presence of a conjugate at Rf 0.31 and another substance at Rf 0.19. The former is probably ascribed to the conjugate of *p*-hydroxybenzoic acid and the latter to that of *p*-hydroxybenzaldehyde. Experiments with rat liver slices showed other two spots, namely a faint spot at Rf 0.16 probably the sulfate conjugate of 3,4-dihydroxybenzaldehyde and a fairly dark spot at Rf 0.11. These results shows that *p*-hydroxybenzaldehyde is easily converted to *p*-hydroxybenzoic acid, either with rat liver supernatant or with slices.

Results with p-Cresol—Experiment with rat liver supernatant showed two spots in the radioautogram, the spot at the smaller Rf value at 0.42 being fainter. The spot ranging from 0.46 to 0.60 of Rf value showed the same Rf value as the synthesized sulfate conjugate of *p*-cresol, determined by the retarded color reaction with 2,6-dibromoquinone-4-chlorimide after hydrolysis with hydrochloric acid. Another substance with smaller Rf value at 0.42 may be ascribed to the conjugate of *p*-hydroxybenzyl alcohol.

Experiments with liver slices showed further conjugates just the same as the case of *p*-hydroxybenzaldehyde with liver slices. This shows that in the supernatant the metabolism of *p*-cresol is almost blocked before *p*-hydroxybenzaldehyde, but with slices further changes proceeded.

DISCUSSION

Sulfate Conjugates of p-Hydroxybenzoic Acid and Its Metabolites—In the rabbit some 6–8 per cent of the administered *p*-hydroxybenzoic acid has been reported to be excreted as an ethereal sulfate (*8*, *4*). In the present study it has been shown that a part of the substance was also conjugated with sulfate in the rat liver slices. The Rf value of the sulfate conjugate of *p*-hydroxybenzoic acid was 0.31 and that of phenol was 0.39 (*9*). The Rf value of the sulfate conjugate of 3,4-dihydroxybenzoic acid was 0.22 and that of *o*-dihydroxybenzene was 0.29. This shows that carboxyl group introduced to the para-direction of hydroxyl group of the aromatic ring reduces the Rf value of the sulfate conjugates with the solvent of acetic acid and butanol. A part of *p*-hydroxybenzoic acid has been reported to be excreted as 3,4-dihydroxybenzoic acid in rabbit by Bray *et al.* (*2*). As there was no conjugate at the position of 0.22 Rf value with the liver supernatant, the hydroxylation of the aromatic ring of *p*-hydroxybenzoic acid seemed to be difficult to occur with the supernatant. But in the case with liver slices, the substance at the Rf value of 0.22, though faint, may be concluded from its Rf value to be the sulfate conjugate of 3,4-dihydroxybenzoic acid. As the amount of the ortho-directing hydroxylation from amino or hydroxyl group of aniline, phenol and chlorophenols was meagre with rat liver (*9*, *10*), and the carboxyl group was almost innert, the hydroxylation of *p*-hydroxybenzoic acid was supposed to occur in the direction of ortho from hydroxyl group and to be meagre. This showed a good agreement with the result of the above experiment. The conjugation of carboxyl group with glycine in the rat has been proved by Rittenberg and Schoenheimer (*12*) and *p*-hydroxyhippuric acid has been isolated by Bray *et al.* (*2*) in the urine of rabbits dosed with *p*-hydroxybenzoic acid. And as the Rf value of the sulfate conjugate of salicylamide was 0.14 and that of salicylic acid 0.39, that of *p*-hydroxyhippuric acid was supposed to be close to that of salicylamide. The substance at 0.11 of Rf value may be ascribed to the sulfate conjugate of *p*-hydroxyhippuric acid, but further studies are needed on this point.

Sulfate Conjugation Related to p-Hydroxybenzaldehyde—Aromatic aldehydes containing a phenolic group is known to reveal a tendency of the aldehyde group to be excreted unoxidised (Ref. 1, p. 87), but *p*-hydroxybenzaldehyde has been reported to be changed to *p*-hydroxybenzoic acid very easily (*4*).

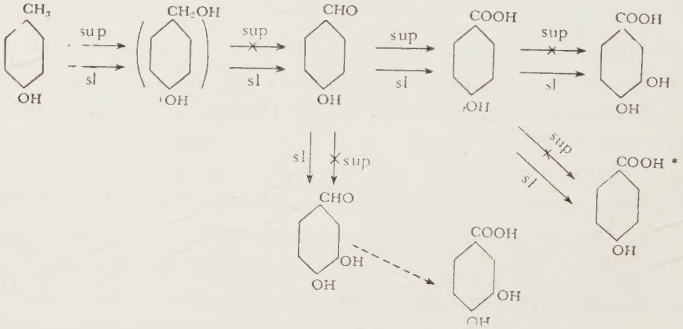
From the present work, the oxidation of the aldehyde group of *p*-hydroxybenzaldehyde proceeded considerably, and showed the spot of the sulfate conjugate of *p*-hydroxybenzoic acid even in the case with the supernatant. The substance which showed the Rf value of 0.16 seems to be 3,4-dihydroxybenzaldehyde analogous in the case with *p*-hydroxybenzoic acid. The Rf value of *p*-hydroxybenzaldehyde was smaller than that of *p*-hydroxybenzoic acid.

Sulfate Conjugation Related to p-Cresol—Neuberg *et al.* (*10*) have isolated from the urine of dogs fed with *p*-cresol a double barium salt of *p*-cresylglucuronide and *p*-cresylsulfuric acid. *p*-Cresol has been reported to be oxidised to *p*-hydroxybenzoic acid (*3*). However the intermediates have not yet been isolated. In the present work of *in vitro* experiments, *p*-cresylsulfate was produced in the supernatant and the sulfate conjugate of *p*-hydroxybenzoic acid was produced in liver slices through *p*-hydroxybenzaldehyde. There seemed to occur another intermediate between *p*-cresol and *p*-hydroxybenzaldehyde, and its sulfate conjugate appeared at 0.42 of Rf value. This substance is probably identical with *p*-hydroxybenzyl alcohol. Benzyl alcohol is known to be oxidised *in vivo* to benzoic acids very easily, but in the case with the liver supernatant the oxidation of *p*-cresol was almost stopped at the stage of the intermediate.

It was concluded from the studies by the aid of paper chromatography of S^{35}-sulfate conjugation, that the oxidation of $-CH_3$ group of *p*-cresol in rat liver proceeded through the probable intermediate $-CH_2OH$ and -CHO, to -COOH. However, with the supernatant of rat liver homogenate the change from $-CH_2OH$ to -CHO was difficult and that from -CHO to -COOH was easy to occur.

The relation of these successive members of a reaction chain was just the same as in the case of the reports by Stanier (*14*, *15*) on the metabolism of aromatic compounds, studied by the method of simultaneous adaptation to the intermediate using fluorescent pseudomonads.

The results of the present investigation from the view point of sulfate conjugation are summarized as follows:



sup.: by the supernatant of the liver homogenate
sl.: by liver slices
-×→ not metabolized to
——→ metabolized to
* modification of -COOH probably by glycine conjugation

SUMMARY

1. A study has been made of the sulfate conjugation of *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde and *p*-cresol in rat liver by the aid of S^{35}-sulfate. Paper chromatography and radioautography has been used to examine the conjugates.

2. There has been found various sulfate conjugates related to the above substances.

3. It has been proved that *p*-cresol is oxidized to *p*-hydroxybenzoic acid through *p*-hydroxybenzaldehyde in the rat liver.

There seemed to exist another intermediate, *p*-hydroxybenzyl alcohol, as a precursor of *p*-hydroxybenzaldehyde.

4. The steps from $-CH_3$ to a substance supposed to be $-CH_2OH$ and from -CHO to -COOH were shown to proceed even in the supernatant of the rat liver homogenate.

5. The occurrence of the sulfate conjugate of 3,4-dihydroxybenzoic acid, from *p*-hydroxybenzoic acid with rat liver slices, was concluded, from its Rf value, though its amount was meagre.

6. Another sulfate conjugate has been found in the metabolite of *p*-hydroxybenzoic acid with liver slices, and this will probably be the sulfate conjugate of *p*-hydroxyhippuric acid.

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STUDIES ON CONJUGATION OF S^{35}-SULFATE WITH PHENOLIC COMPOUNDS

IV. METABOLISM OF *o*-CRESOL, *m*-CRESOL, SALICYLALDEHYDE, SALICYLIC ACID, TOLUENE, BENZOIC ACID AND RELATED SUBSTANCES IN RAT LIVER*

By TOKURO SATO, TAEKO SUZUKI
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(Received for publication, March 10, 1956)

The *in vivo* experiments on the fate of *o*-cresol, salicylaldehyde, salicylic acid and their related substances have been studied by various workers. They have been reviewed by Williams in "The detoxication mechanisms" (*1*). Since then several studies have been made by Bray, Thorpe and *et al.* (*2–4*).

In the previous report of *in vitro* experiments (*5*) with rat liver slices, it was shown that, while salicylaldehyde was conjugated with sulfate fairly well, salicylate did not. There have been no reports of *o*-cresol forming salicylic acid (*Ref. 1*, p. 74), and this point is interesting comparing with the previous experiments about the *p*-hydroxybenzoic acid formation from *p*-cresol *via* *p*-hydroxybenzaldehyde *in vitro* (*6*).

In the present study, the relation between those *o*-hydroxyl derivatives were studied using radioactive sulfate conjugation thechniques, as reported in the previous paper about the *p*-hydroxyl substances (*6*).

EXPERIMENTAL AND RESULT

The experiments were conducted as those in the previous report (*6*).

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* An account of the work described in this paper was presented at the 75th meeting of the Japanese Biochemical Society in Kanto District at the University of Tokyo on 12 March 1955.

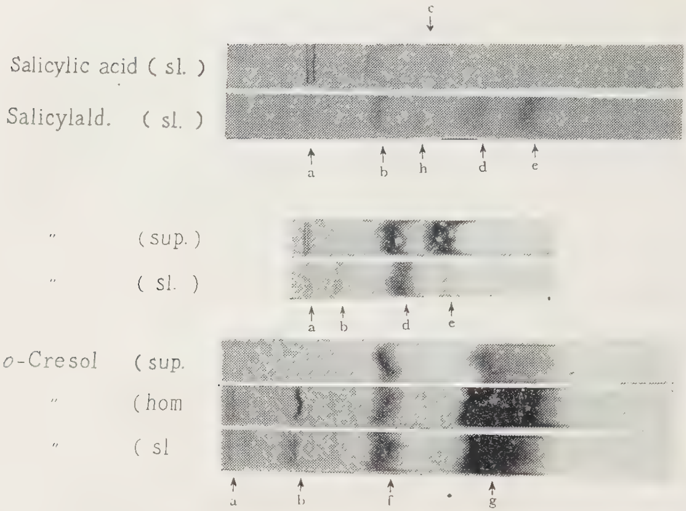


FIG. 1. Sulfate conjugates of salicylic acid salicylaldehyde and *o*-cresol.

Incubation with the supernatant: Supernatant (5%) 0.1 ml.+K ATP 1.5 mg.+20 μg. of the substrate+S^{35}-sulfate, shaken in air phase at 37.5° for 1 hr.

Incubation with the homogenate: Homogenate (50%) with KCl-Ringer-phosphate-succinate 0.1 ml.+K ATP 0.5 mg.+20 μg. of the substrate+S^{35}-sulfate, shaken in O^2 at 37.5° for 1 hr.

Incubation with liver slices: Liver slices 0.1 g.+Ringer-phosphate-succinate 0.1 ml.+20 μg. of the substrate+S^{35}-sulfate, shaken in O_2 at 37.5° for 1 hr.

After the deproteinazation with trichloracetic acid, inorganic S^{35}-sulfate was removed with $BaCl_2$ and nonisotopic SO_4.

Paper chromatography was conducted by the solvent of CH_3-COOH, H_2O, *n*-butanol (1:1:4) for 16 hours.

a. Inorganic sulfate
b. Sulfate conjugate of hydroxybenzoic acid modified at carboxyl group probably by glycine conjugation
c. Sulfate conjugate of 2,5-dihydroxybenzoic acid
d. Sulfate conjugate of salicylaldehyde
e. Sulfate conjugate of salicylic acid
f. Sulfate conjugate of 2,5-dihydroxytoluene
g. Sulfate conjugate of *o*-cresol
h. Sulfate conjugate of 2,5-dihydroxybenzaldehyde (probably)

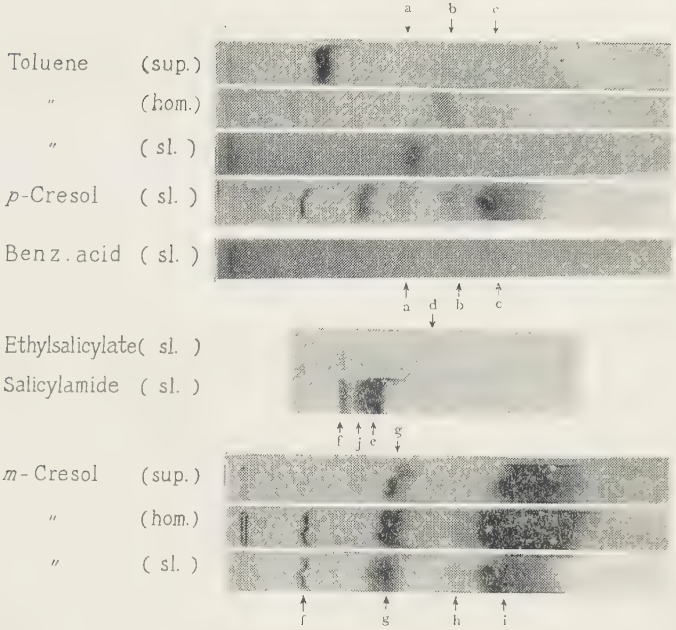


FIG. 2. Sulfate conjugates of toluene, benzoic acid and *m*-cresol. Conditions were the same with Fig. 1.

a. Sulfate conjugate of *p*-hydroxybenzoic acid
b. Sulfate conjugate of *p*-hydroxybenzyl alcohol (probably)
c. Sulfate conjugate of *p*-cresol (probably)
d. Sulfate conjugate of ethylsalicylate
e. Sulfate conjugate of salicylamide
f. Sulfate conjugate of hydroxybenzoic acid modified at carboxyl group probably by glycine conjugation
g. Sulfate conjugate of 2,5-dihydroxytoluene
h. Sulfate conjugate of *m*-hydroxybenzyl alcohol (probably)
i. Sulfate conjugate of *m*-cresol
j. Sulfate conjugate of amide of 2,5-dihydroxybenzoic acid (probably)

The solvent employed for the paper chromatography was a mixture of *n*-butanol, acetic acid, and water (4 :1 :1).

Results with Salicylaldehyde—From the radioautogram, the metabolites of salicylaldehyde showed two spots of S^{35}-sulfate conjugates with rat liver supernatant. The substance at Rf 0.39 showed the same Rf value with the synthesized sulfate conjugate of salicylic acid, which was detected by the color reaction with 2,6-dibromoquinone-4-chlorimide after hydrolysis with hydrochloric acid.

Another substance at Rf 0.30 was likely to be the conjugate of salicylaldehyde. The Rf value of this substance was smaller than that of the conjugate of salicylic acid as in the case with other *p*-hydroxyl derivatives (*6*).

The chromatographic pattern with rat liver slices showed four spots, two of them being common to those with the supernatant. The substances at the Rf value of 0.11 was common to the sulfate conjugates of salicylic acid with liver slices. The last one at 0.17 seemed to be the conjugate of 2,5-dihydroxybenzaldehyde.

Results with Salicylic Acid—Salicylic acid showed no sulfate conjugation with the supernatant, but showed three spots with the slices. There was no spot at the position of the same Rf value with the sulfate conjugate of salicylic acid. The substance at Rf value of 0.21 was likely to be the conjugate of 2,5-dihydroxybenzoic acid (gentisic acid) considered from its Rf value. The substances at Rf 0.11 and 0.12 seemed to be the conjugates of the substances modified at the carboxyl group of salicylic acid and 2,5-dihydroxybenzoic acid as in the case with *p*-hydroxy benzoic acid.

Result with o-Cresol—The pattern of paper chromatography was peculiar in that there was no spot at the position of the Rf value with the conjugate of salicylic acid.

There was a wide range of radioautogram at the Rf values from 0.45 to 0.6 and this agreed with the synthesized *o*-cresyl sulfate. A very faint spot at 0.43 with liver slices seemed to be the conjugate of saligenin but this conversion was quite meagre. The substance at 0.29 with liver slices, the homogenate and the supernatant was likely to be 2,5-dihydroxytoluene interpreted by its Rf value.

Results with m-Cresol—The chromatographic pattern showed that it produced several substances which were conjugated with S^{35}-sulfate. The pattern resembled that of *o*-cresol with liver slices. The conjugate at Rf 0.29 seemed to be the conjugate of 2,5-dihydroxytoluene. In addition, *m*-cresol produced a spot, which was likely to be the conjugate of *m*-hydroxybenzyl alcohol, with liver homogenate and slices, at Rf 0.42.

Results with Several Other Substances—Salicylamide showed a major spot at 0.14 and ethylsalicylate at 0.28. Toluene showed a distinct spot at the position of the conjugate of *p*-hydroxybenzoic acid with liver slices. Another experiment with liver homogenate or its supernatant showed some other conjugates mainly at the position of the sulfate conjugate of hydroxybenzyl alcohol and faintly at the position of sulfate conjugate of a cresol. Benzoic acid showed a faint but clear spot at almost the same Rf value with the conjugate of *p*-hydroxybenzoic acid.

DISCUSSION

Metabolism of o-Cresol Comparing with p-Cresol—Since saligenin is known to be converted to salicylic acid (*Ref.* 1, p. 82) and salicylic acid did not form the ethereal sulfate, the conversion of *o*-cresol to saligenin seemed to be improbable, or if any, the quantity would be very small. In the present study the spot for the conjugate of the substance, which was supposed to be saligenin, was meagre even with liver slices. *o*-Cresol seemed to be hydroxylated at the aromatic ring and the product to be 2,5-dihydroxytoluene as discovered by Preusse (*7*) and confirmed by Bray *et al.* (*4*).

Since -CH_3 group attached to the aromatic ring made the Rf value of the sulfate conjugate increase and the Rf value of the sulfate conjugate of *p*-dihydroxybenzene was 0.19, it was considered that this conjugate at 0.29 was the conjugate of 2,5-dihydroxytoluene. The Rf value of this substance was equal to the metabolite with *m*-cresol, and this was another proof of the identification. As para-hydroxylation from amino or hydroxyl group of phenol, aniline and chlorophenols (*8, 9*) was easier than ortho-hydroxylation in the rat liver, the production of 2,5-dihydroxytolene from *o*-cresol seemed to be easier than that of 3,4-dihydroxytoluene from *p*-cresol (*6*), and the former transformation proceeded even in the supernatant. Further metabolism was not clear but there was a sulfate conjugate at 0.21. It showed almost the same Rf value with the conjugate of 2,5-dihydroxybenzoic acid, and if this was the substance, 2,5-dihydroxytoluene seemed to be oxidized.

The Metabolism of Salicylic Acid—Since 2,5-dihydroxybenzoic acid has been reported to be excreted by the dog dosed with acetylsalicylic acid (*10*) and by the rat (*11*) and rabbit (*2*) dosed with salicylate, it is reasonable to think that salicylic acid would result the spot of sulfate conjugate of 2,5-dihydroxybenzoic acid. As the Rf value of the sulfate conjugate of phenol and that of salicylic acid showed almost the same value, the carboxyl group introduced to the ortho-direction of hydroxyl group of the aromatic ring may not change the Rf value of the sulfate conjugate markedly. The substance at Rf 0.21 showed almost the same value with the sulfate conjugate of *p*-dihydroxybenzene and this substance was interpreted to be the conjugate of 2,5-dihydroxybenzoic acid. There was no sign of the conjugate of salicylic acid.

The conjugate of salicylate with glycine has been confirmed by Bray *et al.* (*3*). The sulfate conjugates at the Rf values of 0.11 and 0.12 may be the conjugate of the substances modified at carboxylgroup of salicylic acid and 2,5-dihydroxybenzoic acid as in the case of *p*-hydroxybenzoic acid. Further studies are needed on those substances.

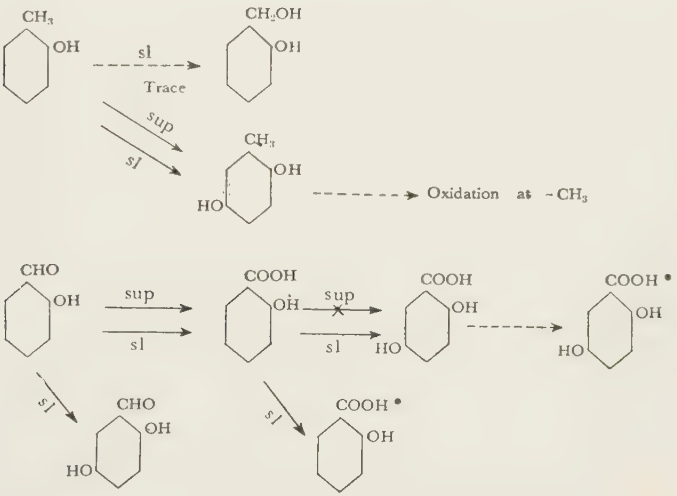
The Metabolism of Salicylaldehyde—Main metabolite of salicylaldehyde in the cat, dog and rabbit has been known to be salicylic acid (*Ref. 1*, p. 87). Since salicylic acid itself was not conjugated with sulfate, the fact, that there appeared the sulfate conjugate of salicylic acid in the course of the metabolism of salicylaldehyde with rat liver, could be explained by the assumption that the conjugation of salicylaldehyde with sulfate precedes the oxidation of aldehyde group. This sort of metabolism has a possibility to take place with other substances.

On the Metabolism of Ethyl Salicylate and Salicylamide—Salicylamide has been reported by various workers to be excreted by dogs and rabbits as an ethereal sulfate, as reviewed by Williams (*Ref. 1*, p. 84) and the degree of hydrolysis has been proved to be 4–7 per cent by Bray *et al.* (*2*). In the present study the substance at the Rf value of 0.14 seemed to be the sulfate conjugate of salicylaminde and the substance at the smaller Rf value at 0.11 was common to the sulfate conjugate of salicylate with liver slices. This substance would be interpreted to be the conjugate of the metabolite of salicylate which was produced by hydrolysis. Just the same interpretation would be applicable to the sulfate conjugates with ethylsalicylate in the rat liver.

The Metabolism of m-Cresol—*m*-Cresol showed very resembling pattern of chromatography with *o*-cresol. Since the para-position of hydroxyl group of *m*-cresol is not occupied the para-hydroxylation from the hydroxyl group may easily take place, comparing with the ortho-hydroxylation of *p*-cresol. Furthermore, *m*-cresol has been reported to be converted to 2,5-dihydroxytoluene by Bray *et al.* (*4*) in the rabbit. The sulfate conjugate at 0.29 Rf value was common to the conjugate of *o*-creseol and the substance was interpreted to be the conjugate of 2,5-dihydroxytoluene, as discussed about the metabolite with *o*-cresol.

In addition, there appeared a conjugate, which was likely to be the sulfate conjugate of *m*-hydroxybenzyl alcohol, with liver homogenate and liver slices. About its minor metabolites, further studies are needed.

The Sulfate Conjugation with Toluene and Benzoic Acid—Toluene is one of benzene derivatives which carry *o,p*-directing groups for which no phenolic metabolites have been reported (*12*). This was interpreted as that side-chain modification *in vivo* may account for the absence of cresols during the metabolism of toluene which is rapidly converted to benzoic acid for which no biological hydroxylation has yet been proved (*13*). However, in the present study, there were distinct spots with toluene, and this shows that the hydroxylation of toluene or its metabolites had a possibility to take place in the rat liver. Considering by their Rf values, they are likely to be the conjugates of *p*-hydroxybenzoic acid, a hydroxybenzyl alcohol and a cresol, though the latter was faint. The sulfate conjugate of a metabolite of benzoic acid in liver slices showed almost the same Rf value with that of *p*-hydroxybenzoic acid, but fainter than those with toluene. The hydroxylation of toluene may take place in itself or in the course of its metabolism to benzoic acid.



sup.: by the supernatant of the liver homogenate
sl.: by liver slices
-×→ not metabolized to
——→ metabolized to
------→ probably metabolized to
* modification of -COOH probably by glycine conjugation

It was difficult to decide the direction of hydroxylation at this stage of investigation, but it is probable that it will take place at *para*- and a small amount at *ortho*-direction of -CH_3 group of toluene and its metabolites.

The results of the present investigation from the view point of sulfate conjugation are summarized as follows:

SUMMARY

1. A study has been made of the sulfate conjugation of *o*-cresol, *m*-cresol, salicylaldehyde, salicylic acid, toluene, benzoic acid and the related substances in rat liver using S^{35}-sulfate. Paper chromatography and radioautography have been adopted to examine the conjugates.

2. There have been found various sulfate conjugates related to the above substances. The identities of the conjugates were deduced or interpreted from a comparison of their Rf values with those of reference or related compounds.

3. It has been shown that most of the o-cresol added could not be metabolized to salicylaldehyde and a considerable part was hydroxylated at the *para*-direction of hydroxyl group. Only a trace of *o*-cresol was thought to be converted to saligenin with liver slices.

4. Salicylic acid in itself was not conjugated, but when oxidized to 2,5-dihydroxybenzoic acid, it was conjugated.

5. Salicylaldehyde was conjugated with sulfate and, when conjugated, it was oxidized to the conjugate of the salicylic acid even with the supernatant of rat liver homogenate.

6. The sulfate conjugation of ethyl salicylate and salicylaminde has been demonstrated.

7. Toluene gave distinct conjugates and benzoic acid a faint one. Toluene seemed to be hydroxylated in the course of its metabolism but its quantity was small.

8. *m*-Cresol was easy to be hydroxylated to 2,5-dihydroxytoluene and also showed a spot which could be interpreted as the sulfate conjugate of *m*-hydroxybenzyl alcohol with rat liver homogenate and slices.

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The Journal of Biochemistry, Vol. 43, No. 4, 1956

EFFECT OF TRYPTOPHAN AND ANTHRANILIC ACID UPON ADRENALECTOMIZED RAT

II. STUDIES ON THE PROTEIN METABOLISM

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(Received for publication, March 16, 1956)

In the proceeding paper, it was reported by Yahito Kotake and Inouye (*1*) that adrenalectomized rats survivable only 4–12 days after the operation could prolong their lives for about one month, when tryptophan or anthranilic acid was orally administered.

In pursuit of the cause, it was found that the liver glycogen content in adrenalectomized rats was markedly reduced at the early post operation state, whereas in the case of administration of tryptophan or anthranilic acid it remained unchanged even six days after the oreration, nearly as high glycogen as in normal healthy rats. The findings seem to do with the prolongation of life caused by the anthranilic acid. In the further experiments such a beneficial effect of anthranilic acid has been confirmed also on protein metabolism, the details of which will be given here.

EXPERIMENTAL AND RESULTS

Male rats weighing about 150 g. were fed for more than 2 weeks on a synthetic casein diet (casein 22 per cent, agar-agar 3 per cent, butter 10 per cent, starch 52 per cent, McCollum salt mixture 6 per cent, yeast 2 per cent and sugar 5 per cent).

They were divided into 4 groups (A, B, C and D), each group comprising of 5 rats. A group served as control. The rats of B group were adrenalectomized, the suprarenal glands of both side being extirpated simultaneously under ether anesthesia. Those of C group were adrenalectomized and then orally administered daily with 2.5 mg. of anthranilic acid. Those of D group were administered daily with 15 mg. of tryptophan.

Effect of Tryptophan or Anthranilic Acid upon the Excretion of the Creati-

nine and Urea in the Urine of Albino Rats—The 24-hours urines were collected from each of 4 groups. The creatinine content in the urine was determined by the Folin's method (*2*) and the urea content by the Rappaport's method (*3*). The mean creatinine value of 5 rats belonging to each group is shown in the Fig. 1. It will be noted that the creatinine content in the urine of adrenalectomized rats, was drastically reduced day after day untill their death, while this was not the case at all with the adrenalectomized rats which followed by the administration of tryptophan or anthranilic acid.

The urea content in the urine of each group was measured at fixed intervals and the mean value of urea is shown Fig. 2.

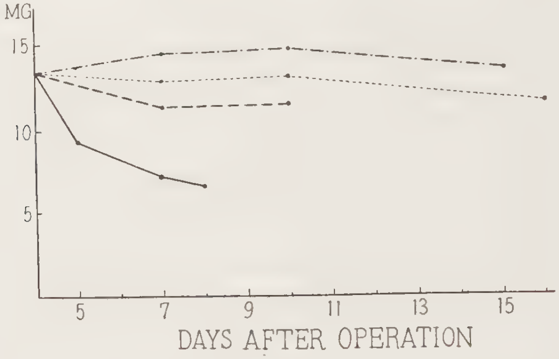

FIG. 1. Mean value of 5 rats.

A -·-·-·- Normal
B ——— Adrenalectomized
C - - - - - Adrenalectomized and administered with anthranilic acid
D ······· Adrenalectomized and administered with tryptophan

Here, the same tendency is to be observed as in the case of creatinine.

Effect of Anthranilic Acid upon the Non-Protein Nitrogen Content in the Blood of Adrenalectomized Rats—On the adrenalectomized rats that were followingly treated with anthranilic acid as in the following experiments, the non-protein-nitrogen content in blood was determined by Rappaport's method (*4*). As shown in the Table I, it was remarkably increased as a result of adrenalectomy, but upon oral administration of anthranilic acid, it was reduced almost to the normal value.

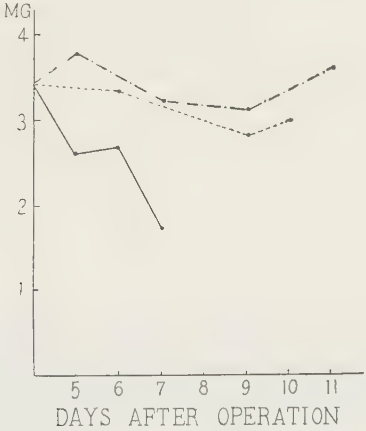

Fig. 2. Mean value of 5 rats.
A —·—·—· Normal
B ——— Adrenalectomized
C - - - - - - Adrenalectomized and administered with anthranilic acid

TABLE I

Non-Protein Nitrogen Content in Blood
(mg. %)

	Normal rat	Adrenalectomized rat	Adrenalectomized rat and administered with anthranilic acid
1	31.89 (180 g.)	7th day 95.55 (180 g.)	7th day 65.04 (185 g.)
2	55.51 (280 g.)	8th day 96.60 (160 g.)	8th day 67.08 (165 g.)
3	33.54 (170 g.)	7th day 81.74 (190 g.)	14th day 81.04 (185 g.)

( ) body wt.

Effect of Anthranilic Acid upon RNA in the Liver—This was examined histochemically by Shibatani's method (*5*). The results indicated that the protein metabolism was much disturbed by adrenalectomy, but the administration of anthranilic acid was capable of readjusting it.

This action of anthranilic acid is quite in accord with the postulation held by Yashiro Kotake (*6*) that the acid displays an important role as biocatalyser in animal body. Since liver concernes in protein metabolism and especially RNA in liver cell is considered to be a site where protein biosynthesis takes place as depicted by Casperson (7), Brachet (*8*) and others, the authors carried out the histochemical examination of RNA in the liver of rats adrenalectomized, and further treated with anthranilic acid. RNA was stained by 0.5 per cent thionine in the liver slice cut out on the 4th day after adrenalectomia and examined microscopically. One of typical figures is illustrated here.

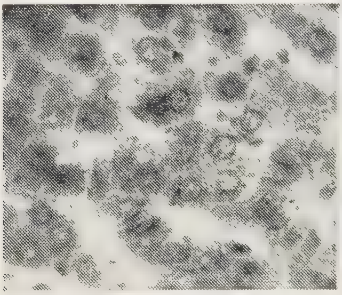
Fig. A

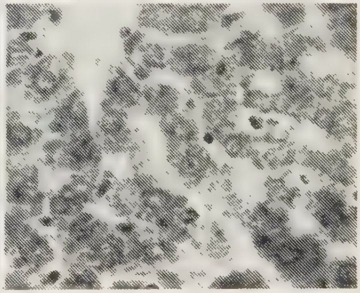
Fig. B

Fig. A shows a picture of a liver slice of the adrenalectomized rat, and Fig. B that of the animal adrenalectomized and subsequently administered daily with 2.5 mg. of anthranilic acid. It will be noted RNA granulae in Fig. A are smaller in size and not clearly outlined compared with those in Fig. B. The result will afford evidence that anthranilic acid exerts a beneficial effect on liver function, which has been lowered by the extirpation of suprarenal glands.

We heartily thank Prof. Yashiro Kotake for his pertinent advices and guidance and heartily thank to Prof. Keizo Kodama for his revision and also heratily thank the Department of Anatomy of our College and Medical School of Osaka University for their help given us in the histopathological examination of RNA.

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The Journal of Biochemistry, Vol. 43, No. 4, 1956

STUDIES ON XANTHURENIC ACID

XIII. EFFECT OF 5-HYDROXYANTHRANILIC ACID ON THE FORMATION AND ON ITS CONJUGATING REACTION OF XANTHURENIC ACID IN ALBINO RAT

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(Received for publication, March 16, 1956)

As reported by Y. Kotake *et al.* (*1*) in the preceeding paper of this series, simultaneous administration of sodium butyrate and tryptophan in fairly large quantities to albino rat caused the excretion of xanthurenic acid in the urine, and at the same time the animal showed symptoms of pancreatogenic diabetes. The symptoms could be, however, supressed by insulin (*2*) and vitamin B_6 and more effectively by methionine (*3*).

The present paper deals with an interesting finding in this connection that 5-hydroxyanthranilic acid, a tryptophan metabolite which was postulated by Y. Kotake (*4*) to be one of biocatalysors, has not only an inhibitory effect upon the formation of xanthurenic acid in the body of an albino rat, but also enhances its conjugation with sulfuric acid.

EXPERIMENTAL

Experiment I

Effect of 5-hydroxyanthranilic Acid on the Formation of Xanthurenic Acid and the Development of Diabetic Symptoms in An Albino Rat Administered with Sodium Butyrate and Tryptophan.

Albino rats fed for more than two weeks on a casein diet and weighing around 200 g. were used. They were divided into 2 groups each comprising of 5 rats. One group was orally administered with 0.4 g. of sodium butyrate and 0.1 g. of tryptophan and served as the reference one. Another groups was similarly treated except further injection of two doses of 0.2 mg./kg. of 5-hydroxyanthranilic acid. The 24-hour urine was collected from each group and subjected to the quntitative determination of xanthurenic acid applying the Glazer-Mueller's method (*5*). As shown in Table I, the amount of excreted xanthurenic acid remarkably decreased in the urne of the test group, in comparison with the reference group. It is evident, therefore, that 5-hydroxyanthranilic acid can remarkably diminish the formation and excretion of xanthurenic acid in the body.

TABLE I

Excretion of Xanthurenic Acid

Expt. no.	Control group	Test group
	mg.	*mg.*
1	9.8	2.1
2	8.24	1.4
3	7.52	1.85
4	8.19	2.38
5	7.73	3.6
6		1.8
7		1.22
8		2.5
9		2.4
10		1.52
Average	8.29	2.07

Blood Sugar—The measurement was conducted by the King-Garner's method (*6*) with the animals of each group. The results are shown in Fig. 1.

The blood sugar curve of the control rats showed the quite similar tendency to that in the previous report by Y. Kotake and Inada (*1*), namely the initial hyperglycemia, and subsequent hypoglycemia, which followed, 24 hours later, by the continued hyperglycemia. In the second hyperglycemia period, sugar was detectable in the urine and diabetic symptoms were noticeable. In the test group, however the blood sugar was found to fluctuate more or less at the initial state, but it was restored to normal level, 24 hours later, without developing any diabetic symptoms at all.

Paper-Partition-Chromatography—The above experiment indicates that 5-hydroxyanthranilic acid may block the excretion of xanthurenic acid in the rat administered with both sodium butyrate and tryptophan as the result which no continued hyperglycemia develops. To get more intimate knowledge on the action of 5-hydroxyanthranilic acid on the tryptophan metabolism in the presence of sodium butyrate, paperchroma-

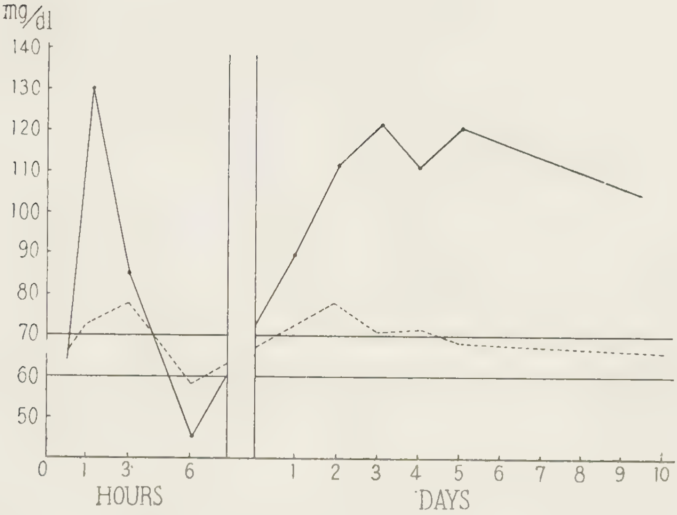

FIG. 1. Blood sugar curve.
——— Control rat (Na butyrate 0.4 g. + tryptophan 0.1 g.)
------- Test rat (Na butyrate 0.4 g. + tryptophan 0.1 g. + 5-hydroxy anthranilic acid)

tographical examination was carried out on the urine. For this purpose one-dimensional ascending method was used.

As the developer a mixture of methano, *n*-butanol, benzene and water (2 :1 :1 :1) was employed. The developing time was 15 hours.

On each sheet of the filter paper more than 10 spots were obtained. The identification of each spot and its rough quantitative assumption were attempted on the basis of Rf value, fluolescent color, color-reaction and ultraviolet absorption spectrum.

3-Hydroxykynurenine, its conjugated compounds and xanthurenic acid were found to be decreased remarkably in the urine of the test group compared with those in the reference group. On the other hand, fluorescent spots were identificable to be the conjugated compounds of xanthurenic acid, glucuronide of anthranilic acid and its derivatives, along with a fairly large amounts of N^{α}-acetylkynurenine. It can be concluded, therefore, that the alterations of tryptophan metabolism caused by the administration of sodium butyrate were subsided by 5-hydroxyanthranilic acid.

Quantitative Determination of Kynurenic Acid—The above-stated role of 5-hydroxyanthranilic acid is quite similar to that of insulin as reported by Kotake, Kotake, Hishikawa, Sakan and Yamaguchi (*2*). In order to raise more precise evidence the amount of kynurenic acid excreted in the urine of both groups were determined.

The fluorescent spots corresponding to kynurenic acid from the paper-chromatogram of the urine were adjusted to pH 8.0, cut out and extracted with phosphate buffer (pH 7.4). On this extract the amount of kynurenic acid was estimated according to the Knox's method (*7*) at 332 mμ with use of Beckmann spectrophotometer. The results are shown in Fig. 2.

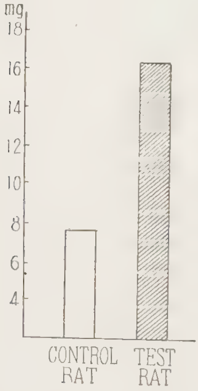

Fig. 2. Excretion of kynurenic acid (mean value of 3 rats)

In comparison with the urine of the reference group, kynurenic acid in that of the test group increased remarkably by the action of 5-hydroxyanthranilic acid.

Experiment II

Effect of Sodium Butyrate on the Conjugating Reaction of Xanthurenic acid.

Albino rats fed for more than two weeks on a casein diet and weighing around 200 g. were divided into two groups, one group served as the control another was further force-fed with 0.4 g. of sodium butyrate. To each groups 10 mg. of xanthurenic acid (m. p. 296.5°) in ammoniacal solution (pH 7.2–7.4) was injected subcutaneously, and the 24-hour urine was collected.

On this urine quantitative determination of ethereal sulfate of xanthurenic acid (*8*) was carried out. This was conducted in accordance with the Sperber's method (*9*) measuring turbidity due to to barium sulphate by Beckmann's spectrophotometer at the wave length of 650 mμ. The results are summarised in Table II, which shows clearly that sodium butyrate diminished the conjugation of xanthurenic acid with sulphuric acid. Since xanthurenic acid may enter into another forms of conjugation, it has been intended to determine total amout of conjugated xanthurenic acid. The collected urine was mixed with conc. HCl until the final concentration of the acid became 5 *N* and subjected to hydrolysis by heating in a sealed tube at 100°. The cortents of xanthurenic acid both before and after the hydrolysis were determined by the aforementioned Glazer-Mueller's method. As shown in Table III, the percentage of conjugated xanthurenic acid was

TABLE II

Excreted Ethereal Sulfate

(mean value of 3 rats)

	Excreted ethereal sulfate pro 10 mg. xanthurenic acid injection	
	*The amount of SO_3	Conjugating xanthurenic acid with H_2SO_4
	mg.	%
10 mg. xanthurenic acid injected group	0.8877	22.74
Oral administration with 0.4 g. sodium butyrate and xanth. acid injection	0.415	10.63

* This indicates the absolute increased amount of conjugated SO_4 by in jection of 10 mg. xanthurenic acid.

TABLE III

Quantitative Determination of Xanthurenic Acid

(mean value of 3 rats)

		Hydrolysis		Conjugated xanth. acid	Percentage of conjugation to excretion of total xanth. acid	Relative per cent of conjugated xanth. acid
		Xanth. acid				
		Before hydrolysis	After hydrolysis			
1	Xanth. acid (inj.)	6.02*mg.*	8.52*mg.*	2.5*mg.*	29.3%	100%
2	Na butyrate (*per os*)+xanth. acid (inj.)	6.53	7.44	0.91	12.2	36.4
3	Methionine (inj.)+Na butyrate+xanth. acid. (inj.)	5.95	7.50	1.55	20.6	62.0
4	5-hydroxyanthraniclic acid (inj.)+Na butyrate+xanth. acid (inj.)	5.95	7.67	1.72	22.4	68.8

found to be about one-third when sodium butyrate was administered to the animal.

Experiment III

Effect of Methionine and 5-Hydroxyanthranilic Acid on the Conjugating Reaction of Xanthurenic Acid.

A lot of the rats treated in the same ways as described in *Expt. II* was further injected subcutaneously with 10 mg. of methionine and 24 hours later again with 10 mg of methionine plus xanthurenic acid. Another lot of the rats received injection of 100 μg. of 5-hydroxyanthraninlic acid instead of methionine in the above procedures.

On the urine collected from each lot, the amount of conjugated xanthurenic acid were determined as usually. The results are indicated in Table III. It is apparent from the Table that methionine as well as 5-hydroxyanthranilic acid can increase the conjugating action of xanthurenic acid which is suppressed by sodium butyrate.

DISCUSSION AND SUMMARY

From the experiments stated above, it is now clear that 5-hydroxyanthranolic acid, a tryptophan metabolite postulated by Y. Kotake as one of biocatalysors, acts inhibitory to the output of xanthurenic acid. Both methionine and 5-hydroxyanthranilic acid are capable of restoring the conjugating reaction of xanthurenic acid weakened by administration of sodium butyrate in the body.

From these findings, it is possible to conclude that 5-hydroxyanthranilic acid inhibits the formation of xanthurenic acid and accelerates its conjugating reaction and as the results the accumulation of xanthurenic acid in the body and the appearance of diabetic symptoms are prevented.

We wish to thank heartily to Prof. Yashiro Kotake, for his advice and revision and also heartily thank to Prof. K. Kodama, for his revision.

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The Journal of Biochemistry, Vol. 43, No. 4, 1956

INFLUENCE OF ADRENOCORTICOTROPIC HORMONE (ACTH) ON THE BIOSYNTHESIS OF CORTICOSTEROID IN VITRO

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(Received for publication, March 19, 1956)

It has been established that adrenocorticotropic hormone (ACTH) stimulates corticosteriod biosynthesis in adrenal tissue *in vitro* (*1–5*). Haynes *et al.* (*1*) showed that ACTH stimulated the incorporation of C^{14} from acetate-1–C^{14} into hydrocortisone. Stone and Hechter (*5*) reported that the action of ACTH was concerned with a step involved in the conversion of cholesterol to progesteron.

However, there have been few reports that described the quantitative change of each corticosteroid. This report describes the experiments carried out in our laboratory showing that ACTH increases the formation of Δ^4-3-ketosteroid, especially hydrocortisone, in beef adrenal *in vitro*.

EXPERIMENTAL

Incubation—Adrenal glands were obtained at slaughter house and brought back to the laboratory in icecold isotonic saline solution. Then, the glands were freed from adhering fat and medulla, and the cortical tissues were sliced to about 1 mm thickness by free hand. 2 to 3 g. of slices were placed in Erlenmeyer-flasks containing 10 to 15 ml. of Krebs-Ringer-phosphate (*6*)-glucose solution (pH 7.4; glucose 0.2 per cent). Incubation was carried out at 37.5° for 2.5 hours in 100 per cent oxygen, with mechanical shaking. ACTH was added at 2 to 3 units (ACTHAR, Armour) per flask.

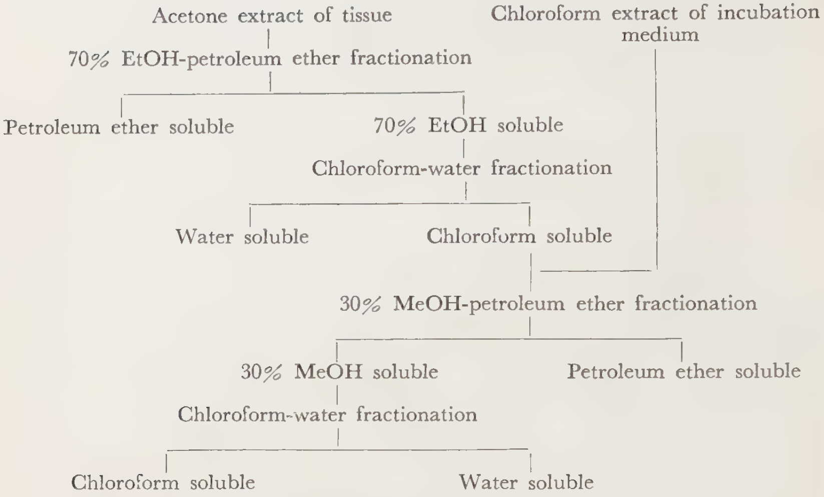
In the experiments with homogenate, adrenals freed from adhering tissues were homogenized with Krebs-Ringer-phosphate-glucose solution (2 ml./g.) in Waring blendor. A 5 ml.-portion of this homogenate was placed in a Warburg vessel and incubated as described above.

Quantitative Analysis of Total Corticoid Produced—After incubation, the slices were homogenized and extracted with acetone, and the incubation media were extracted with chloroform. Acetone extract and chloroform extract were fractionated by the method similar to that of Brady (*7*), as shown in Fig. 1.

The residue of the chloroform was dissolved in the reagent grade methanol and

FIG. 1

Procedure of Fractionation


Acetone extract of tissue
Chloroform extract of incubation medium
70% EtOH-petroleum ether fractionation
Petroleum ether soluble
70% EtOH soluble
Chloroform-water fractionation
Water soluble
Chloroform soluble
30% MeOH-petroleum ether fractionation
30% MeOH soluble
Petroleum ether soluble
Chloroform-water fractionation
Chloroform soluble
Water soluble

Chloroform was washed with 1% Na_2CO_3, *N*/2 HCl and water then dried over Na_2SO_4.

the optical density of this solution was determined at 225, 240 and 255 mμ. The absorption at 240 mμ owing to the Δ^4-3-ketonic grouping was calculated by the method of Allen (*8*). In this procedure, 80 per cent of cortisone was recovered.

Paper Chromatography—After removal of the methanol by vacuum distillation, the residue was dissolved in a minimal volume of methanol-chloroform (1:1) and applied to a paper chromatography according to Burton *et al.* (*9*), using toluene-propylene glycol as a solvent.

The filter papers with four narrow limbs, as shown in Fig. 2 were used in this experiment and washed throughly with methanol. The corticoid solutions were applied to the starting line and developed for 48 hours. The fractions which run off the papers were concentrated and reapplied to the second strips, and then developed for 16 hours with the same solvent. The effluents from the second strips were concentrated and applied to the third strips, and then developed for 4 hours. The authentic samples of cortical hormones were applied to the same paper and developed simultaneously. For the 48 hours run, 50 μg. of hydrocortisone and cortisone were used. 100 μg. of 11-desoxy-17-hydroxycorticosterone (Substance S) for the 16 hours run and 100 μg. of desoxycorticosterone for the 4 hours run were employed as controls.

After drying, the strips were bisected perpendicularly and one was used for the detection of corticosteroid with TPTZ (triphenyltetrazolium chloride) reagent. The other was used for quantitative analysis.

RESULT

Influence of ACTH on Corticosteroid Production by Cortical Slices

As shown in Table I, total corticosteroid production increased serveral times after incubation only and markedly increased after incubation with ACTH.

TABLE I

Influence of ACTH on Corticosteroid Production by Cortical Slices

Exp. No.	I Initially present (Non-incubated)		II Incubated (No ACTH)		III Incubated with ACTH	
	Tissue used (*g.*)	Corticoid produced (*μg.*)	Tissue used (*g.*)	Corticoid produced (*μg.*)	Tissue used (*g.*)	Corticoid produced (*μg.*)
1	13	46	7.5	75.4	7.5	126
2	15	0	8	32.8	5	74.4
3	18	22.6	8	46.2	4	53
4	22	37.2	8	89	4	64
5	30	121.5	10	42.8	6	68.8
6	23	10.9	10	78	6	63
7	40	43.1	8	57.2	4	45.5
8	54	134.5				
Total	215	415.8	59.5	421.4	36.5	494.7
Average	1.9 *μg./g.*		7.1 *μg./g.*		13.5 *μg./g.*	

The paperchromatograms of I, II, and III are shown in Fig. 2.

From these chromatograms, it is supposed that fractions 2, 3, and 6 are considered to be hydrocortisone, cortisone and desoxycorticosterone respectively. To determine whether the fraction 4 is 11-desoxy-17 hydroxycorticosterone (Substance S) or not, absorption spectrum of sulfuric acid chromogen (*10*) was measured. As shown in Fig. 3, sulfuric acid chromogen of fraction 4 had absorption maxima at 285 and 375 mμ and differed from those of Substance S which showed maxima at 285 and 535 mμ. From the absorption data, this substance is con-

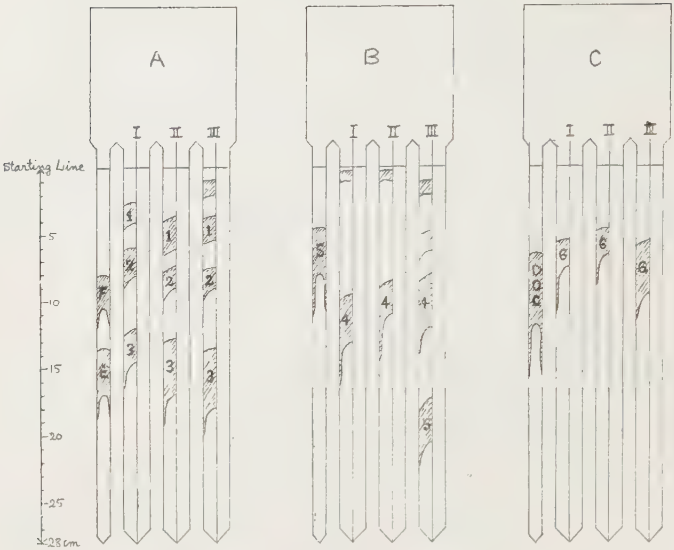


FIG. 2. Paperchromatogram of Adrenal Extract.
A: 48 hours run, 350 ml. of solvent in reservoir.
B: 16 hours run, 200 ml. of solvent in reservoir.
C: 4 hours run, 200 ml. of solvent in reservoir.
I, Non incubated. II, Incubated (No ACTH). III, Incubated with ACTH.

sidered to be corticosterone.

To determine the quantities of these steroids, the zones corresponding to each spot were cut off from the half section of the chromatograms and the steroids were eluted with ethanol. Evaporating the solvent in a vacuum, the residue was dissolved in methanol. The steroid content in the methanolic solution was estimated by the absorption at 240 mμ, using the calculation of Allen (*8*) and Elliot *et al.* (*11*). The result is shown in Table II.

The amounts of hydrocortisone, cortisone and corticosterone produced per 100 g. of adrenal cortical tissue are shown in Fig. 4.

As shown in Fig. 4, hydrocortisone, cortisone and corticosterone increased 2.7, 2.4, and 2.3 times, respectively, by incubation only and increased 7.8, 3.8 and 4.8 times, respectively, after the incubation with ACTH.

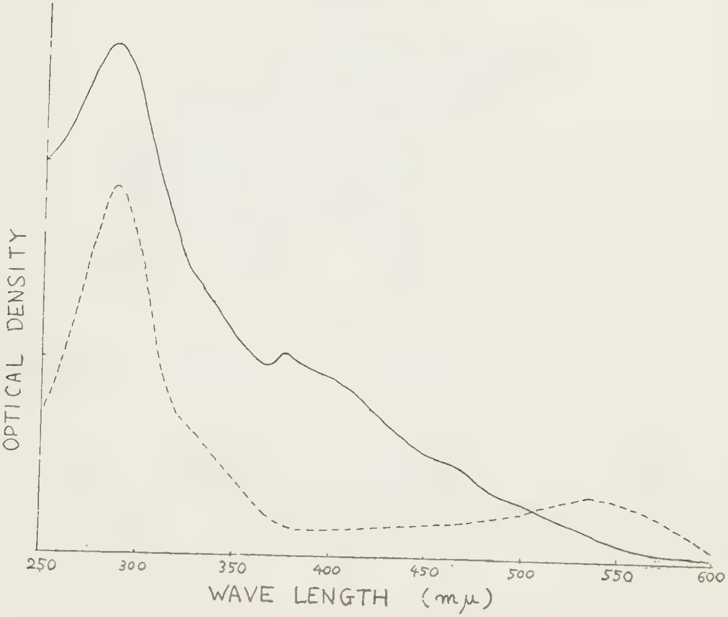


Fig. 3. Adsorption Spectrum of Sulfuric Acid Chromogen of Fraction 4 and Substance S.
Solid line, Fraction 4.
Dotted line, Substance S.

Influence of ACTH on Corticosteroid Formation in Adrenal Homogenate

As shown in Table III, total corticosteroid formation increased 3 to 4 times only by incubation, but ACTH had no influence on corticosteroidogenesis. In this case, however, oxygen uptake increased by addition of ACTH.

DISCUSSION

It has been demonstrated by Hechter *et al.* (*5*) (*12*) that ACTH stimulates the earlier step involved in the conversion of cholesterol to progesterone and that the ratio of hydrocortisone to corticosterone produced was constant. In the present studies, the ratio of hydrocortisone to corticosterone is 0.62 initially, 0.75 after incubation and 1.0

TABLE II

Amount of Each Corticosteroid*

Fraction	I Non-incubated (μg.)	II Incubated (No ACTH) (μg.)	III Incubated with ACTH (μg.)
Hydrocortisone	24.7	18.5	32.8
Cortisone	38.8	25.8	25.3
Corticosterone	39.2	24.6	32.2
Fraction 5		23.6	24.6
Desoxycorticosterone	17.6	12.5	7.4

Calculation: $\left[OD_{240}-\frac{OD_{225}+OD_{255}}{2}\right]\times$ Converting Factor $=\mu$g./ml. in MeOH

Converting factor: Hydrocortisone, 55; Cortisone 56; Corticosterone, 53; Desoxycorticosterone, 44; Fraction 5 (Compound A?), 50.

* Eluted from the half section of strip.

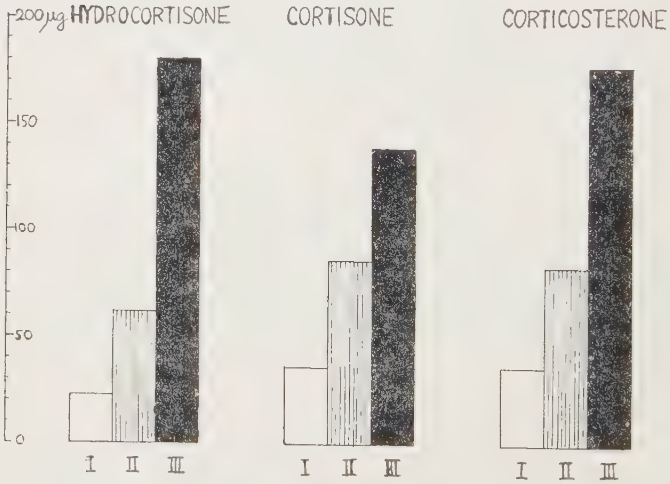


FIG. 4. Ratio of Hydrocortisone, Cortisone and Corticosterone in 100 gm. of Tissue.

I: Non incubated. II: Incubated (No ACTH). III: Incubated with ACTH.

TABLE III

Influence of ACTH on Corticosteroid Formation in Adrenal Homogenate

Exp. No.	I Non-incubated corticoid extracted (μg./g.)	II Incubated (No ACTH) corticoid produced (μg./g.)	III Incubated with ACTH corticoid produced (μg./g.)		
1	4.7	14.3	0.2 I.U.* 16.1	0.4 I.U.* 12.0	0.6 I.U.* 15.3
2	3.9	17.0	0.6 I.U.* 19.0	1.2 I.U.* 16.2	3.0 I.U.* 12.9

* Added amount of ACTH per vessel.

after incubation with ACTH. From this it is supposed that the biosynthesis of hydrocortisone was increased by ACTH. Thus, it may be assumed that ACTH stimulates the pathway not only in conversion of cholesterol to progresterone, but also in conversion of progesterone to hydrocortisone.

It is interesting that the corticosteroid biosynthesis in homogenate was not influenced by ACTH and that the amount of corticosteroid produced in homogenate was as much as in slices incubated with ACTH. Hechter also described that ACTH acted only on the preparations containing intact cells (*13*).

The result that corticosteroid biosynthesis in homogenate was not influenced, but oxygen uptake increased by the addition of ACTH, seems to indicate that ACTH has some effect other than that in corticosteroidogenesis.

SUMMARY

The influence of ACTH on corticosteroid biosynthesis in beef adrenal in vitro was studied and the following result was obtained.

1. ACTH increased the corticosteroid biosynthesis in beef adrenal slices.

2. The major fractions produced were hydrocortisone, cortisone and corticosterone, and hydrocortisone was remarkably increased by the addition of ACTH.

3. In adrenal homogenate, the corticosteroid biosynthesis was

not influenced by ACTH.

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DENATURATION AND INACTIVATION OF ENZYME PROTEINS

I. BACTERIAL PROTEINASE METHOD FOR THE DETERMINATION OF THE RATIO OF DENATURATION OF GLOBULAR PROTEINS

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It has been suggested that most of enzymes belong to globular proteins and the enzyme action seems to depend on the specific internal structure of their molecules which are stabilized by a great number of weak secondary intramolecular bonds. Though the denaturation of a globular protein is thought to arise from an alteration of this intramolecular structure, it is not easy to distinguish the denatured state of a protein from the native one, because it can be conclusive only according to the experimental criteria such as 1) decrease in solubility, 2) increase in reactivity of constituent groups, 3) changes in physical properties, 4) loss in biological activity, and 5) increase in susceptibility to enzymatic hydrolysis. Many methods belonging to the second and third criteria are useful for the determination of the " degree of denaturation "—extent of the alteration of the intramolecular structure —, but they are not always suitable for the determination of the " ratio of denaturation "—quantitative distinction of the denatured state of a protein from the native one—, because these changes do not always proceed according to an " all or none " reaction in the course of denaturation. The first criterion is very convenient to distinguish the denatured protein from the native one, but it is only available as a quantitative method in a few cases when the protein becomes completely insoluble by denaturation.

In the case of enzyme proteins, the 4th criterion is the most useful for determination of the " ratio of denaturation." However, some enzyme proteins are sometimes inactivated without denaturation by the action of some specific inhibitors which combine with a few essential groups in the protein molecule (see succeeding papers, especially the 4th report of this series). Therefore, it is required for studies on various modification processes of the enzyme proteins to adopt another criterion which indicates the alteration of intramolecular structure of the proteins in addition to the 4th criterion.

To comply with the above requisite, a convenient method answering to the 5th criterion has been established which is useful for the quantitative determination of denatured and native protein of various globular proteins separately. The increase in susceptibility of denatured proteins to trypsin and other proteinases has been pointed out by Linderstrøm-Lang *et al.* (*1*) and Tazawa (*2*), and used for the criterion of denaturation of several proteins (*3–8*). However, these methods are not convenient and accurate for determination of the "ratio of denaturation," since the procedures and properties of proteinases used in these methods are not suitable. On the contrary, the bacterial proteinase (*9*, *10*) crystallized by us from the culture medium of *Bac. subtilis* possess suitable properties in many respects for the above purpose (*9*, *11*). In the present investigation, it has been experimentally established that the proteolytic method using the bacterial proteinase is able to determine the "ratio of denaturation" most precisely.

MATERIALS

Proteinases—Crystalline bacterial proteinases were prepared from the cultures of two strains of *Bac. subtilis*, strain R and N′, by the method using an ion exchange resin (*9*, *10*), and lyophilized after two recrystallizations. Each proteinase will be abbreviated as BPR and BPN′. Crystalline trypsin and chymotrypsin prepared by the method of Kunitz and Northrop (*12*) were kindly furnished by Dr. N. Sakota of this University.

Enzyme Proteins—Crystalline bacterial α-amylase and Taka-α-amylase (amylase of *Aspergillus oryzae*) were prepared by the method of Hagihara (*9*) from a culture of *Bac. subtilis* (strain N′) and the method of Akabori, Hagihara, and Ikenaka (*13*) from Takadiastase-Sankyo, respectively.

METHODS

1. Assay for Proteinase Activity—Casein digesting activity was measured by Kunitz's method (*14*) modified by Hagihara (*9*).

2. Assay for Amylase Activity—Starch dextrinize activity was measured by Wohlgemuth's method modified by Hagihara (*9*), using a standard solution for the exact determination of the end point.

3. Preparation of Denatured Proteins—Solution of completely urea-denatured amylases was obtained by heating an amylase solution containing 6 *M* urea at 60° or 70° at pH 7–8 until the complete loss of enzyme activity. Solution of partially denatured amylases was obtained by treating with 4 M urea at 40° to the desired ratio of inactivation. Completely heat-denatured solution was obtained by heating the enzyme solution of pH 7.5–8.0 at 80°–90° until the complete loss of activity. Taka-α-amylase did not coagulate by heat-denaturation at the pH above 7.0. Bacterial amylase was partially precipitated by heat-denaturation at neutral pH, but the protein precipitated could be digested completely by the bacterial proteinases. The amylase was denatured without coagulation by heating in the solution above pH 8.0 containing almost no salts. Acid- or alkali-denaturation was carried out by treating the solution with dilute phosphoric acid or sodium hydroxide at 60°.

4. Method for the Examination of the Course of Digestion of Proteins—Ten ml. of about 0.6 per cent solution of enzyme protein was mixed with 20 ml. of the proteinase solution containing 0.1 M phosphate buffer, pH 7.5, and the mixture was kept at 30°. Concentration of BPN′ in the above proteinase solution was about 0.015 per cent—0.01 per cent in the reaction mixture—and the concentrations of BPR (*ca.* 0.015 per cent), trypsin (*ca.* 0.03 per cent), and chymotrypsin (*ca.* 0.1 per cent) were adjusted to show the same activity as that of BPN′ solution. In the cases of trypsin and chymotrypsin, phosphate buffer was added just before use, because they are stable only in acidic solution. At optimal intervals 3 ml. of the above reaction mixture was poured into 2 ml. of 1 M trichloroactic acid (TCA) and resulting suspension was filtered through a toughened filter paper after standing for 30 minutes or more.

Concentration of the digestion products in the TCA filtrate was measured by the optical density (OD) at 278 mμ or by the color of Folin reaction at 660 mμ. In the former method, the filtrate was diluted to 3- or 6-folds with distilled water, if necessary, and in the latter method, 1 ml. of the filtrate was mixed with 5 ml. of 0.45 M sodium carbonate and 1 ml. of 5-fold dilution of Folin-Ciocalteu reagent (*15*), then the mixture was incubated at 40° for 20 minutes. As the blank test, 1 ml. of the protein solution was added to the mixture of 2 ml. of the proteinase solution and 2 ml. of 1 M TCA, and then treated as above.

5. Assaying for the "Ratio of Denaturation"—In the present investigation, the following procedure was used, although several modified methods have been used in the series of this kind of study and some of them have already been reported elsewhere (*9, 11*). One ml. of the test solution was added to 2 ml. of the proteinase solution, which contained 0.1 M phosphate buffer, pH 7.5, and had activity about 3 times stronger than that of the solution used in *Method 4*. After the mixture was incubated at 30° for 15 minutes, 2 ml. of 1 M TCA was added and then treated as for *Method 4*. At the same time, solution of native and completely denatured protein of the same concentration as the test solution were treated as above. The "ratio of denaturation"—abbreviated as "RD"—of the test solution was calculated from the following equation:

$$\text{“RD” } (\%) = \frac{\mathrm{OD}_{\text{test}} - \mathrm{OD}_{\text{nat.}}}{\mathrm{OD}_{\text{den.}} - \mathrm{OD}_{\text{nat.}}} \times 100$$

where, $\mathrm{OD}_{\text{test}}$, $\mathrm{OD}_{\text{nat.}}$ and $\mathrm{OD}_{\text{den.}}$ show the optical density (at 278 mμ or Folin color) of the filtrate from the above digested solution, to which was added TCA of the test, native and completely denatured protein, respectively.

RESULTS

Experiment 1

Digestion of Native and Completely Denatured Bacterial Amylase with Four Proteinases.

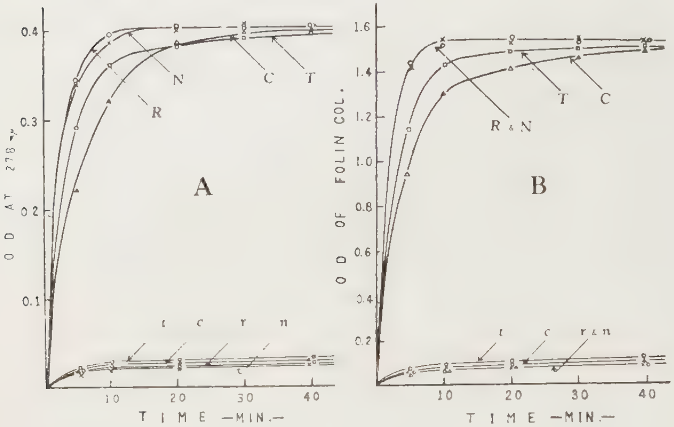

Fig. 1. Digestion-courses of native and urea-denatured bacterial amylase with 4 kinds of neutral proteinases.

Concentration of digestion product soluble in 0.4 *M* TCA was measured by optical density at 278 mμ in Fig. A or by optical density at 660 mμ of Folin colour (1/7) in Fig. B.

Curve N, R, C and T show the digestion-courses of the denatured protein with BPN′, BPR, chymotrypsin and trypsin respectively, and n, r, c and t show these of the native protein.

As shown in Fig. 1, when native bacterial amylase was incubated with BPR, BPN′, trypsin, and chymotrypsin in neutral pH, a very slight amount of digestion product was detected. Since these digestion products were hardly increased and the activity of the amylase was not lost in the course of proteolytic action, it seems to be attributed to the contamination of a small quantity of denatured protein. In the case of trypsin or chymotrypsin, a slight amount of autodigestion product may be added to them, because these proteinases are unstable in a neutral solution. On the contrary, the denatured amylase was rapidly digested with all proteinases. However, a noteworthy difference was found between the courses of digestion of the bacterial proteinase and the other two proteinases. As shown by Curves N and R in Fig. 1, the denatured amylase was easily digested to the completely soluble state in TCA by the bacterial proteinases and the amount of digestion product rapidly attained a constant value, while it was difficult to find the amount of digestion product arriving at a constant value in the case of trypsin and chymotrypsin. To show above facts more clearly, the difference between the amount of digestion product from native and that from denatured bacterial amylase ($OD_{den.}-OD_{nat.}$) produced by the action of four proteinases at various time are listed in Table I.

TABLE I

Differences between the Amount of Digestion Product from Native and that from Urea-denatured Bacterial Amylase by the Action of Four Proteinases at Different Time of Reaction

Methods	Time \ Proteinase	BPR	BPN′	Trypsin	Chymotrypsin
		Diff. between native and denatured state ($OD_{den.}-OD_{nat.}$)			
278 mμ	*min.* 5	0.335	0.326	0.270	0.203
	10	0.365	0.368	0.342	0.305
	20	0.379	0.378	0.348	0.356
	30	0.388	0.371	0.347	0.376
Folin	5	0.927	0.897	0.705	0.591
	10	0.956	0.967	0.891	0.832
	20	0.977	0.954	0.917	0.892
	30	0.962	0.953	0.947	0.939

Experimental conditions are described under Fig. 1.

Experiment 2

Digestion of Native and Completely Denatured Taka-α-amylase with Four Proteinases.

As shown in Fig. 2, when native and heat-denatured Taka-α-amylase were treated as above, fundamentally the same results were obtained as in the case of bacterial amylase.

Experiment 3

Digestion of Bacterial and Taka-α-amylase Denatured by Different Methods with Bacterial Proteinase.

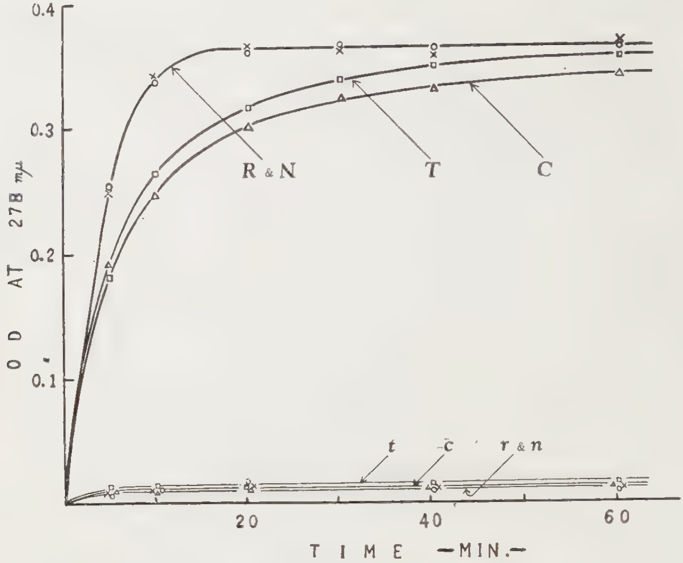

FIG. 2. Digestion-courses of native and heat-denatured Taka-α-amylase with 4 proteinases.

The experimental procedures were the same in the case of Fig. 1, but the results obtained by 278 mμ-method were only shown in the figure.

The symbols in the figure are also the same as Fig. 1.

Though it was clear from *Experiments 1* and *2* that urea-denatured bacterial amylase and heat-denatured Taka-α-amylase were completely digested with the bacterial proteinase and the concentration of digestion product measured by the two methods rapidly attained a constant value, the following experiments were carried out to see whether or not the same results would be obtained in the case of the proteins denatured by different procedures.

As shown in Table II, the same concentration of digestion product was produced from the same concentration of bacterial amylase irrespective of denaturing procedures, such as urea treatment at 60° or 70° or by heat treatment at 80° or 90°.

In the case of Taka-α-amylase, the same results were obtained by urea or heat denaturation, independent of the assaying methods such

TABLE II

Concentration of Digestion Product Produced from Bacterial Amylase Denatured Completely by Different Ways by the Action of BPR

Condition of denaturation	Concent. of amylase (%)*	Native one ($OD_{nat.}$)**	Denatured one ($OD_{den.}$)	Difference ($OD_{den.}-OD_{nat.}$)	Diff. per concn. ($OD_{diff.}/\%$)
6*M* urea, 70°,60′	0.269	0.071	0.515	0.444	1.65
6*M* urea, 60°,60′	0.269	0.071	0.505	0.434	1.61
pH 8.0, 90°,30′	0.269	0.051	0.500	0.449	1.67
6*M* urea, 70°,30′	0.313	0.047	0.570	0.522	1.67
6*M* urea, 60°,60′	0.313	0.047	0.562	0.515	1.65
pH 8.0, 80°,30′	0.313	0.029	0.545	0.516	1.65

All values were measured by *Method 5* using Folin reaction.

* Concentration of amylase was calculated from amylase activity before the denaturation was carried out.

** The values contain all blank values such as Folin-positive substances contained in TCA, those of TCA-soluble impurities of the proteinase and amylase, in addition to digestion product of native amylase preparation. In the digestion of native samples to be compared with urea-denatured proteins, corresponding amount of urea was added after the native protein was mixed with the proteinase.

TABLE III

Digestion of Taka-α-amylase Denatured Completely by Various Ways.

Condition of denaturation	By Folin color reaction (1/7)			By 278mμ(1/6)
	Native ($OD_{nat.}$)	Denatured ($OD_{den.}$)	Difference ($OD_{den.}-OD_{nat.}$)	Difference ($OD_{den.}-OD_{nat.}$)
6*M* urea, 60°,30′	0.045	0.448	0.443	0.149
pH 6.0, 75°,30′	0.027	0.470	0.443	0.146
M/45 H_3PO_4, 60°,30′	0.026	0.460	0.434	0.143
M/30 NaOH, 60°,30′	0.025	0.462	0.437	0.148
M/30 NaOH, 60°,60′	0.025	0.464	0.439	0.149

About 0.28% solution of the amylase was treated by *Method 5* (see Table II).

as Folin or ultraviolet absorption (at 278 mμ) methods, as shown in Table III. However, acid-denatured Taka-α-amylase showed a somewhat smaller value. This imcompleteness of digestion was assumed to arise from a slight renaturation occurring in neutralization at the instant before digestion was taken up (see Paper III of this series (*16*)).

Experiment 4

Susceptibility of Partially-denatured Taka-α-amylase to Bacterial Proteinase.

To see whether the susceptibility of denatured part of a partially denatured protein is different from that of a completely denatured protein or not, following two kinds of Taka-α-amylase solutions (A and B) were digested with weak BPR (about 1/3 of *4*) and the course of digestion was measured by the dialysis of a solution of Taka-α-amylase ($D^{30'}_{40^\circ}=9{,}000$) aganist a neutral buffer solution after denaturation had occurred in the presence of 4 M urea at 40° to such a degree that the enzyme lost about 78 per cent of the initial activity (remaining activity $D^{30'}_{40^\circ}=2{,}100$]. Solution B (mixture of native and denatured proteins) was prepared by mixing the native Taka-α-amylase with the same protein which had been denatured completely with urea and dialysed, in the proportion of 78 :22. The concentration of both solutions, A and B, were adjusted to nearly the same.

As shown in Fig. 3, the digestion course of denatured amylase which is included in partially denatured preparation (solution A) was practically the same as that of completely denatured amylase contained in solution B. When Folin method was used for the determination of digestion product, the same result was obtained.

Experiment 5

Comparison between the "Ratio of Inactivation" and the "Ratio of Denaturation" Measured by Three Proteinases.

About 0.5 per cent of Taka-α-amylase was treated with 4 M urea until the enzyme lost 27.7 per cent of its initial activity and the "ratio of denaturation" of this protein was measured by the proteolytic method as in 5 using three proteinases, BPR, chymotrypsin, and trypsin. The results are shown in Table IV. As supposed from the results of *Experiments 1–4*, the "RD" measured by the bacterial proteinase showed the best coincidence with the "ratio of inactivation."

DISCUSSION

Comparison of Various Methods for the Determination of the "Ratio of Denaturation"—Several methods belonging to the second criterion in the

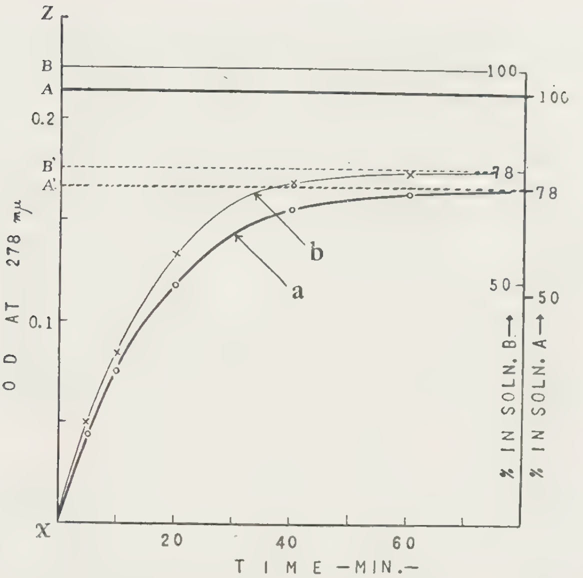

Fig. 3. Digestion-courses of partially denatured Taka-α-amylase and that of mixture contaning Taka-α-amylase completely denatured and native one.

Curves a and b show the digestion courses of the partially denatured protein (soln. A) and above mixture (soln. B) in the presence of BPR. Length of XA and XB show the concentration of total protein in the soln. A and B respectively. XA′ and XB′ show the concentration of the inactivated protein contained in soln. A and denatured protein mixed in soln. B respectively.

introduction have been often used for the determination of the "degree of denaturation" of many proteins, and some of them were thought to be useful for the measurement of the "ratio of denaturation" if the changes measured occurred as "all or none" reaction in the process of denaturation. Therefore, two representative methods among them, amperometeric titration of -SH groups with $AgNO_2$ (*17*) and Biuret reaction in weak alkali (*18*), were tried for the determination of the "RD" of partially denatured Taka- and bacterial α-amylases. However, the former method was not applicable, for both α-amylases gave no test for -SH either before or after denaturation. The latter method gave no quantitative and no reporducible results though denatured

TABLE IV

"Ratio of Denaturation" of Partially Denatured Taka-α-amylase Measured by Three Proteinases

Proteinase	Ratio of inactivation (%)	By 278 mμ			By Folin reaction		
		$(OD_{test.} - OD_{den.})$	$(OD_{den.} - OD_{nat.})$	Ratio of denat.(%)	$(OD_{test} - OD_{nat.})$	$(OD_{den.} - OD_{nat.})$	Ratio of denat. (%)
BPR	27.7	0.245	0.866	28.5	0.080	0.290	28.1
Chymotrypsin		0.240	0.820	29.2	0.082	0.270	30.3
Trypsin		0.265	0.830	31.9	0.078	0.253	30.8

The "ratio of denaturation" of the Taka-α-amylase (about 0.5%) inactivated 27.7% by urea treatment was measured by the proteolysis method (*Method 5*) using above three proteinases.

amylases gave somewhat larger values than native ones. From the above and other results obtained by us, these methods were thought to be applicable only to limited proteins. Among the methods belonging to the third criterion, change of optical rotation has been often used for the study of denaturation, but the method is thought to give only the "degree of denaturation" and not the "RD" of a protein. Furthermore, considerably large amount of protein is required for this method. Therefore, the method seems to be unsuitable as a microassay of the "RD." If denatured protein is insoluble under the conditions in which the native one is soluble, the "RD" of a partially denatured protein will be measurable, as the application of trypsin, chymotrypsin, and pepsin using a salt solution to precipitate the dedenatured proteins by Northrop and Knitz (*8*). Though the denatured Taka- and bacterial α-amylases were precipitated at pH 7–4 and 6–4, respectively, they were difficult to be measured quantitatively by the foregoing method, because they were partially soluble even under such conditions. Furthermore, this method could not be used in the case of urea denaturation in which the denatured protein was highly soluble. Therefore, above precipitation method seems to be applicable only in limited cases. Compared with the foregoing methods, the proteolytic method using the bacterial proteinases—each of BPR and BPN′ is similary usable—is much better for the determination of the "RD," as it can be carried out easily and correctly in a microscale for almost all of the globular proteins tested in various kinds of denaturation.

Procedures for Assaying the Amount of Digestion Products—To determine the amount of denatured protein in the presence of native one by the proteolytic method, it is essential to separate the digestion product (corresponding to denatured proteins) from the undigested (corresponding to native proteins). If the digestion product is soluble in a suitable concentration of TCA in which the undigested protein is completely insoluble, the additions of TCA to the protein solution treated with the proteinase is very convenient for this purpose. For the determination of the amount or concentration of the digestion product separated as above, measurement of nitrogen seems to be useful, but this method is fairly troublesome and requires a fairly large amount of the sample, and is difficult to apply in case the sample contains nitrogenous compounds other than protein, such as urea and guanidine. If the susceptibility of a protein to proteolytic digestion is " all or none " as regards denaturation, *i.e.*, denatured molecules of a protein are completely digested and native ones are not at all, other more convenient methods such as Folin reaction or ultraviolet absorption method (at 278 mμ) can be applied as the nitrogen determination. The results of *Experiments 1–4* show that this " all or none " nature holds, as discussed in the next section.

The " RD " of a partially denatured protein is generally expressed by the Eq. (1). In the case of ultraviolet absorption method (at 278 mμ), Eq. (2) is useful for the calculation of " RD," because the shift of optical density at 278 mμ of many proteins is negligibly small before and after proteolytic digestion. In the case of Folin method, Eq. (3) must be used instead of Eq. (2) since this color intensities are fairly different among native, denatured, and digested state of a protein (ordinary native$<$denatured$<$digested). Even in the case of ultraviolet absorption method, Eq. (3) is more convenient than Eq. (*2*), as all the blank values are included in value $\mathrm{OD}_{\mathrm{nat.}}$ of the former. Furthermore, even if the native protein is slightly digestable, the values may be corrected by using this equation.

$$\text{“ RD ” } (\%) = \frac{\text{Denatured protein}}{\text{Total protein}} \times 100 = \frac{\text{Amount of digestable protein}}{\text{Amount of total protein}} \times 100 \ldots \text{(1)}$$

$$\text{“ RD ” } (\%) = \frac{\text{Corrected OD* of TCA filtrate of digested test solution}}{\text{OD of test solution of the corresponding concentration}} \times 100 \ldots \text{(2)}$$

$$\text{“ RD ” } (\%) = \frac{\mathrm{OD}_{\mathrm{test.}} - \mathrm{OD}_{\mathrm{nat.}}}{\mathrm{OD}_{\mathrm{den.}} - \mathrm{OD}_{\mathrm{nat.}}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots \text{(3)}^{**}$$

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* Since TCA shows a fairly large extinction at 278 mμ and TCA-soluble

impurities in the proteinase often show slight extinction, these values must be corrected by the blank test.

** The symbols of this Equation are explained under the *Method 5.*

" All or None " Nature of Proteolytic Digestion and Relation between Loss of Enzyme Activity and Susceptibility to Proteolysis—From the results shown in *Experiments 1–4*, it is evident that the susceptibility of native or denatured protein to the bacterial proteinase is well accountable by the " all or none " theory. Especially from *Experiment 4*, it is shown that all molecules of denatured amylase contained in a protein partially denatured by a mild treatment are digested as well as the molecule completely denatured by a drastic treatment. It is supposed from this fact that when the secondary intramolecular structure of a native protein is modified to some degree in the process of denaturation, the modified molecule—even if the modification is not large—is hydrolyzed by a slight progress of this process and is further modified through the hydrolysis till the state becomes completely digestable. Thus, differences of susceptibilities among many denatured proteins become negligible unconcerned with the degrees of the denaturation. On the other hand, difference between native and denatured protein can be very large, because the latter is completely attacked by the proteinase but the former not at all.

Though a protein molecule modified to some degree becomes completely susceptible to the proteolytic digestion as mentioned above, it is very important whether the degree of digestable modification is different or not from the loss of enzyme activity. Since the results given in *Experiments 4* and *5* made it clear that the " ratio of inactivation " of a partially denatured Taka-α-amylase agrees well with the " RD " measured by the bacterial proteinase, both degrees of modification of the protein molecule are supposed to be the same or very closely related with one another. Because the same result was obtained in the case of partially denatured bacterial α-amylase, this presumption may be true for many enzyme proteins.

Comparison of Four Proteinases as A Tool for the Determination of " RD "—As both bacterial proteinases, BPR and BPN′, are very stable at pH 6–9.5 (*9*, *19*), contrary to trypsin and chymotrypsin which are unstable at neutral pH and therefore must be kept at lower pH just before use in order to prevent destruction by autodigestion, these enzymes can be preserved in solutions of neutral pH which is used in the determination procedure and are most convenient for handling. Furthermore, autodigestion products from the latter proteinases in the determination procedure may disturb the quantitative determination of digestion products from the test solution, for the autodigestion may occur during the procedure after the substrate (denatured protein) have been consumed.

As shown in the results of *Experiments 1* and *2*, all the foregoing proteinases did not digest native proteins to any remarkable extent, but easily digested the denatured proteins. However, the course of digestion of the latter with bacterial proteinases were somewhat different from that with other two enzymes. With the bacterial proteinases, the amount of digestion products rapidly reached a constant value when measured by ultraviolet absorption or Folin method, but with trypsin or chymotrypsin of the same activity a constant value was not easily reached. Though almost complete digestion may be reached with a very strong activity of trypsin or chymotrypsin for a long time, unfavorable side-reactions may cause autolysis of the proteinase or a slight digestion of the native protein under these conditions. No difference was observed between the course of digestion with two bacterial proteinases, BPR and BPN′. It is supposed that the difference between the bacterial proteinases and other two proteinases resulted from the different specificity of the enzymes. As already reported (*19*), the bacterial proteinase (BPN′) has a very wide specificity as compared with trypsin or chymotrypsin and it can hydrolyse the peptide bonds of casein and gelatin to the extent of about twice that of trypsin. Northrop *et al.* (*6*) studied the digestion of partially denatured trypsin with pepsin and obtained a somewhat good parallelism between the amount of precipitable protein in TCA and remaining activity, but pepsin is only useful for very limited cases, since many enzyme proteins are not stable at acidic pH. They have also reported (*7*) that a fairly good parallelism was obtained between the remaining activity of the enzymes and the amount of residual protein in the duration of autodigestion of trypsin and chymotrypsin at pH 6–9. The parallelism reported by them was, however, not so strict as the results indicated in our experiments. It can be supposed that their comparatively large error must have arisen from the unsuitability of proteinase and assaying methods. As shown in the result of *Experiment 6*, when trypsin or chymotrypsin was used, the error is somewhat larger than in the case of bacterial proteinases even when our convenient assaying method was adopted. From the properties mentioned above the bacterial proteinase is thought to be most suitable for the determination of the “ratio of denaturation” of many proteins.

SUMMARY

It was confirmed that native enzyme proteins, crystalline bacterial and Taka-α-amylase, were not digested by bacterial proteinase, trypsin or chymotrypsin, but the denatured proteins were rapidly digested with such proteinases. With trypsin or chymotrypsin, however, the denatured proteins were difficult to be digested to the state completely soluble in 0.4 M TCA, in which undigested proteins—native proteins—were completely insoluble. With the bacterial proteinases, the digestion products soluble in TCA rapidly reached a constant value measured by ultraviolet absorption or Folin method. The susceptibility of denatured proteins to the proteinase was practically the same whether the denaturation occurred partially or completely. The same concentration of denatured proteins which was produced through various ways gave the same amount of digestion product by the action of bacterial proteinase. Based on the above facts, a convenient and accurate method for determining the " ratio of denaturation "—quantitative determination of denatured and native states—of partially denatured proteins has been presented, in which bacterial proteinase was used to digest a denatured protein and the digestion product was measured by ultraviolet absorption and Folin method after separating from undigested proteins with TCA. When this method was used for partially denatured Taka-α- amylase, the " RD " agreed well with the " ratio of inactivation." Comparison of this method with other methods for determining the " RD " of the protein was also discussed.

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DENATURATION AND INACTIVATION OF ENZYME PROTEINS

II. DENATURATION AND INACTIVATION OF BACTERIAL AMYLASE

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In the previous paper (*1*) a very convenient and accurate method was described for determining the "ratio of denaturation" of partially denatured globular proteins using proteolysis with a bacterial proteinase. By determining the denaturation by the above method and assaying the enzymatic activity, denaturation and inactivation of crystalline bacterial amylase (*2*) in the process of various treatment was studied. Though some results of above investigation have been briefly reported elsewhere (*2*, *3*), more detailed experiments will be described in the present communication.

MATERIALS AND METHODS

Crystalline Bacterial Amylases—Two kinds of *B. subtilis* amylase, BAH and BAN′, were used. BAH was crystallized from a synthetic culture medium of *B. subtilis H* according to the original method of Hagihara (*2*), while BAN′ was crystallized from a commercial preparation of *B. subtilis N′* by his modified method using adsorption on raw starch and decolorization with an anion exchange resin (Duolite A-2).

Crystalline Bacterial Proteinase—BPR (Proteinase of *B. subtilis R* (2)) were used (see the previous report (*1*)).

Assay for Amylase Activity—Beside starch dextrinizing power ($D_{40^\circ}^{30'}$) (see the previous paper), saccharification activity was measured by Fehling-Lehman-School's method as occasion demanded. In the latter case, one unit ($S_{40^\circ}^{10'}$) was represented as the quantity of reducing substances equivalent to 1 ml. of 0.05 *N* $Na_2S_2O_3$ which was liberated from 6 ml. of 1 per cent soluble starch at 40°, pH 6.0 for 10 minutes. The measured values were corrected by the standard curve showing the initial and linear reaction. The test solution was diluted with 0.05 *M* NaCl solution containing 0.01 *M* Na_2HPO_4 in order to prevent inactivation by [dilution. The " ratio of inactivation " (RI) was calculated by the following equation:

$$\text{“ RI ” (\%)} = \frac{\text{Initial activity—Remaining activity}}{\text{Initial activity of native amylase}} \times 100 \ldots\ldots\ldots\ldots \quad (1)$$

Method for Assaying the " Ratio of Denaturation "—Though the procedures were fundamentally the same as those described in the previous paper (*1*), they were slightly modified in the present investigation as follows: One ml. of the test solution was mixed with 2 ml. of the proteinase solution and 1 ml. of 1.6 *M* trichloroacetic acid (TCA) was added to the above mixture after incubation at 40° for 10 minutes. As the proteinase solution, about 0.04 per cent BPR containing 3/40 *M* phosphate buffer solution of pH 7.5 was used and the amount of digestion products in the TCA filtrates was measured only by the Folin method (*1, 2*). The " ratio of denaturation " was calculated as follows (see the preceding paper (*1*)):

$$\text{“ RD ” (\%)} = \frac{OD_{test} - OD_{nat.}}{OD_{den.} - OD_{nat.}} \times 100 \ldots\ldots\ldots\ldots \quad (2)$$

Other procedures will be described in each section of the results.

RESULTS

Experiment 1

Relation between Inactivation and Denaturation in Heat, Acid, and Urea Treatment.

It has been shown that the " ratio of inactivation " of Taka-α-amylase partially inactivated with urea agreed well with the " ratio of denaturation " measured by the proteolytic method (*1*). In the present investigation, these two " ratios " were measured when about 0.3 per cent bacterial amylase (BAN′) was partially (30～60 per cent) inactivated by heat (at 65° and pH 8.5 for 30 minutes), acid (0.02 *M* acetic acid at 10° for 20 minutes) or by urea (5 *M*, at 40° for 2 hours). Even if the protein was precipitated by denaturation as in the case of weak acid treatment, the denatured protein was digested by the proteinase to the completely soluble state. As shown by the results in Table I, a good agreement of the two " ratios," " RD " and " RI," was obtained. Therefore, it is reasonable to conclude that the inactivation of the enzyme by these treatments was caused by the denaturation—alteration of intramolecular structure—of the enzyme proteins and not by a special change of the essential radical in the molecule.

Experiment 2

Inactivation and Denaturation in the Duration of Heat Treatment.

Though the " RI " and " RD " of about halfdenatured BAN′ agreed with each other, the following experiment was carried out to see

TABLE I

Compariscn between " Ratio of Inactivation " and " Ratio of Denaturation of Heat, Acid, and Urea Treated Bacterial Amylase

Treatment		Heat*	Acid**	Urea***
Initial activity	($S_{40°}^{10'}$/ml.)	2,100	2,100	2,100
Residual "	(")	1,150	830	1,360
Lost "	(")	950	1,270	740
Ratio of inactivation	(%)	45.2	60.5	35.2
Folin OD of native enzyme protein	($OD_{nat.}$)	0.035	0.038	0.042
Folin OD of test enzyme protein	($OD_{test.}$)	0.232	0.294	0.207
Folin OD of completely denatured enzyme protein	($OD_{den.}$)	0.477	0.472	0.488
Ratio of denaturation	(%)	44.6	58.6	37.0

* About 0.25 per cent BAN′ solution of pH 8.5 containing no salts was heated at 65° for 20 minutes and 1 ml. of this soln. was used for assaying amylase activity after dilution with 200 ml. of the diluting-solution and another 1 ml. was used for the determination of the " RD " by the proteolytic method.

** Since it was difficult to pipette the test solutions for the determination of the " RD " and " RI " from the treated BAN′ solution on account of precipitation of the denatured BAN′ in the weakly acidic solution, the acid treatment of the enzyme was carried out by a different manner from the case of heat treatment as follows ; 0.5 ml. of the BAN′ solution, concentration of which was twice as much as that in the case of heat treatment, was mixed with 0.5 ml. of *ca.* 0.02 M acetic acid and then the proteinase solution containing excess Na_2HPO_4, for the neutralization of acetic acid in addition to the ordinary buffer action, was added to the mixture after incubation at 10° for 10 minutes. The precipitated protein was completely digested by the proteinase and, therefore, the " RD " of the amylase was determined similarly as in other cases. The " RI " of the BAN′ solution treated quite similarly as above was also measured in parallel with the above experiment.

*** The BAN′ solution of the same concentration as in the case of acid treatment was mixed with the same volume of 10 M urea and incubated at 40° for 2 hours. Assay of the " RI " and " RD " were the same as in the case of heat treatment.

whether or not the same result would be obtained at any degree of denaturation of another bacterial amylase (BAH). When a neutral

TABLE II

Inactivation and Denaturation of A Crystaline Bacterial Amylase (BAH) in the Duration of Heat Treatment

Duration of treat.	Proteolysis	Activity test			Proteolytic method		
		Residual	" RI "	Rate const. (k_1)	Opt. Dens. $(OD_{test.})$	*** " RD "	Reat const. (k_1)
min.		$(S^{10'}_{405}/ml.)$	%			%	
0	—	2,450	0.0				
	+	2,410	0.6		0.053*	0.0	
10	—	1,860	24.1	0.0120			
	+				0.179	24.0	0.0119
20	—	1,440	41.2	0.0115			
	+	1,470	40.0	0.0111	0.273	41.9	0.0118
40	—	1,030	57.9	0.0094			
	+				0.360	58.5	0.0095
60	—	800	67.4	0.0081			
	+	810	67.0	0.0080	0.412	68.2	0.0083
90	—	567	76.9	0.0071			
	+				0.463	78.1	0.0073
120	—	444	81.9	0.0060			
	+	437	82.2	0.0062	0.492	83.6	0.0065
150	—	346	85.8	0.0057			
	+	338	86.2	0.0057	0.509	86.9	0.0059
Completely denatured	—	0					
	+	0	100		0.578**	100	

About 0.3 per cent solution of BAH ($S^{10'}_{405}$/ml. 2450, adjusted to pH 8.5 with NH_4OH, containing no salts) was heated at 63°. At each period during the treatment, 3 lots of test solutions of the same volume (1 ml.) were pipetted from the above solution at the same time and one was immediatly diluted to 500 ml. for the assay of amylase activity of " proteolysis," and the other two were immediately mixed with the proteinase solution. After incubation at 40° for 10 minutes, one of the latter solutions was diluted to 500 ml. for assay of activity of " proteolysis " and the other was treated for the determination of the " RD."

* This value was used as $OD_{nat.}$ for the calculation of the " RD."

** One ml. of the test solution was completely denatured by heating at 80° for hour to obtain the value of $OD_{den.}$

*** Using the above two values, the " RD " was calculated as follows:

$$\text{“ RD ” } (\%) = \frac{(\mathrm{OD}_{\mathrm{test}}) - 0.053}{0.578 - 0.053} \times 100 = \frac{\mathrm{OD}_{\mathrm{test}} - 0.053}{0.525} \times 100$$

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solution of the crystalline bacterial amylase (*2*) was denatured by heat, some part of the denatured protein precipitated, though the amount of the precipitate was varied not only with the kind of amylase used but also with the kind and concentration of salts contained in the solution and also with heating temperature. However, the amylase was denatured gradually without precipitation or with slight homogeneous turbidity, when the slightly alkaline solution of the amylase containing almost no salts was heated at 60°∼65°. In the present experiment, the courses of inactivation and denaturation of BAH were studied by the heat treatment under the above conditions.

As shown in Table II, the “ RI ” measured by the proteolysis method agreed well with one another at every stage in the duration of the heat treatment. Because the rate constants of inactivation and denaturation-reaction calculated as the first order reaction showed very approximate values in earlier period of the treatment, both reactions were concluded to be a unimolecular reaction at first, as in most cases of protein denaturations. When the test solution at any stage during the treatment was incubated with the bacterial proteinase, the activity of the solution was not almost changed before and after the digestion as shown in the table. Therefore, it may be concluded that the active part of the amylase protein remains practically unaffected by the proteolysis at any time.

Fundamentally the same results as above was obtained in the duration of heat treatment of BAN′.

Experiment 3

Inactivation and Denaturation in Urea Treatment.

The inactivating effect of urea on the bacterial amylases of *B. subtilis* was much influenced by the concentration of urea and the temperature. It was also very much influenced by pH in acidic solutions, but almost not at neutral or slightly alkaline solutions. Representative results obtained as to the effect of urea concentration, temperature, and pH on the inactivation of a amylase of *B. subtilis* (BAH) are summarized in Table III. The amylases once inactivated by urea treatment were not reactivated by dialysis as well as the case of heat inactivated amylases which were not reactivated by cooling.

From the results shown in Table III, a convenient condition was chosen to investigate the course of inactivation and denaturation, *i.e.*,

TABLE III

Effects of Various Conditions on Inactivation of A Bacterial Amylase in the Presence of Urea

Effect of	Concn., Temp., or pH	Other conditions*	Activity remaining during the treatment (%)					
			0.5 hrs.	1 hr.	2 hrs.	4 hrs.	6 hrs.	8 hrs.
(A) Concn. of urea (*M*)	0.0	pH 7.0 temp. 40°C		100	100	100	100	100
	1.2			100	98	96	90	88
	2.4			98	96	90	81	77
	4.8			95	87	69	58	51
	7.9			75	47	22	11	7
	9.6			60	31	10	5	3
(B) Temp. (°C)	0	pH 7.0 urea 7.2 *M*	100	98	95	88	84	
	10		98	95	85	75	71	
	20		96	91	80	68	59	
	30		90	78	50	36	31	
	40		57	37	13	3	≒ 0	
(C) pH				Urea 7.2 *M*		(Without urea)		
				1 hr.	2 hrs.	(1 hr.)	(2 hrs.)	
	4.8	40°, urea 7.2 *M* or 40°, no urea		21	4	(66)	(45)	
	5.4			27	8	(86)	(68)	
	6.0			68	42	(95)	(85)	
	6.6			85	62	(100)	(100)	
	7.4			85	66	(100)	(100)	
	8.0			85	64	(100)	(100)	
	9.0			82	64	(100)	(100)	

In the above experiments, about 0.06 per cent solution of BAH ($D^{30'}_{40^\circ}$/ml.=3,000) was treated under the above conditions, and the remaining activity was measured by the dextrinizing powers at various times.

* The treated solutions contained 0.01 *M* Na_2HPO_4-KH_2PO_4 buffer system of pH 7.0 in the experiment (A) and (B), and 0.04 *M* CH_3COOH-CH_3COONa, Na_2HPO_4 or $Na_2B_4O_7$-KH_2PO_4 system of various pH in the experiment (C). Therefore, concentration of the stabilizing metal ion (Na^+) is much larger in the case of (C) than in (A) and (B).

about 0.3 per cent solution of BAH containing 6 *M* urea and 0.01 *M* ammonium phosphate buffer of pH 7.0 was kept at 40°, and inactivation and denaturation was measured during this treatment, similarly as in the case of heat treatment. The results are shown by Curves A, B, and C in Fig. 1. It has been indicated, as in the case of heat treatment, that the loss in activity was quite proportional to the amount of denatured protein measured by the proteolysis method and the remaining activity was not decreased by the proteolysis at every stage in the duration of urea treatment.

Experiment 4

Protective Effect of Calcium.

It has been already reported that one of bacterial amylase, BAA (amylase of *B. subtilis A*), was protected by many metal ions from heat inactivation in the following order: Ca^{++}, Sr^{++}, Na^{+}, Mg^{++}, K^{+}, Ba^{++}, NH_4^{+}. Effect of calcium ion was especially large among them (*2*, 7). As to the protection of calcium ion, almost the same results were obtained in the case of other bacterial amylases such as BAH or BAN′ than BAA. Calcium ion also protected the amylases from inactivation by acid and urea treatment. To show the above facts, some representative results are presented in Table IV.

It has been reported by several workers that calcium ion protects many α-amylases such as pancreas (*4*), malt (*4*, *5*, *6*), and *Aspergillus* (*4*, *8*) amylases. However, β-amylases of sweet potato and malt were not protected by calcium. Therefore, it was supposed that the protection effect of calcium for the α-amylases may be attributed only to the protection of a small essential radical (or part) in the molecule of the enzyme proteins. To see whether above supposition is valid or not, inactivation and denaturation of a bacterial amylase containing calcium ion in the duration of urea treatment were compared with the results of *Expt. 2* in which calcium was not present. As shown by results in Fig. 1, the inactivation and denaturation of amylase proceeded in parallel and followed almost the same time-course as that of the unimolecular reaction, independent of the presence of calcium ion, though the rate of the reaction decreased by its presence. Therefore, it was concluded that the protective effect of calcium ion on the inactivation of the amylase is not owing to the protection of a special radical (or part) of the amylase but to the protection of the enzyme molecule from denaturation as a whole.

TABLE IV

Protective Effect of Calcium Ion on Heat, Acid and Urea Inactivation of BAH

Kind of treatment	Conc. of $CaCl_2$ added (M)	Remaining activity after treatment for					
		0 hr.		1 hr.		3 hrs.	
		($D^{30'}_{400}$)	(%)	($D^{30'}_{400}$)	(%)	($D^{30'}_{400}$)	(%)
(A) Heat at 53°, pH 8.0	0	1100	100	220	20.0	40	3.6
	0.02	1100	100	1040	94.5	940	85.5
(B) Acid* at pH 5.0, 30°	0	1020	100	120	11.8	30	2.9
	0.02	1020	100	1060	104.0	1000	98.0
(C) Urea** with 7.2 M at pH 7.0, 40°	0	1420	100	280	19.7	60	4.2
	0.0002	1420	100	380	26.8	96	6.8
	0.001	1420	100	850	59.9	350	24.6
	0.004	1420	100	1100	77.5	830	58.5
	0.02	1420	100	1150	81.0	870	61.4
	0.1	1420	100	1260	88.6	920	64.8

* About 0.2 per cent BAH was dialyzed against 0.02 M ammonium acetate buffer solution of pH 7.0 for 15 hours in an ice box, and the dialyzed solution was divided into two parts. One of them was used in the experiment of heat treatment (A) after pH was adjusted to 8.0 with NH_4OH, and the other was used in the acid treatment (B) after pH was adjust to 5.0 with CH_3COOH at 0° to 2°.

** Experiment of urea treatment (C) was carried out independently from the above experiment (A and B). In the experiment, 2 volumes of 10.8 M urea solution was added to 1 volume of about 0.08 per cent BAH solution prepared by dissolving BAH in distilled water and a very small quantity of aqueous ammonia to adjust to pH 7.0 at about 2°. Therefore, kind and amount of contaminating metal ions—though they were very small—was somewhat different in the solution of this case (C) from that in (A) or (B).

In every case of (A), (B), and (C), the initial amylase activity (activi tyat 0° time treatment) was measured by diluting rapidly an aliquot of the cold (0° to 2°) test solution and the remaining activities at every time were measured after the solutions were heated at the set temperatutes (53°, 30°, and 40° in (A), (B), and (C), respectively).

Experiment 5

Protective Effect of Starch-like Compounds

It has been believed that some enzymes are protected from inacti-

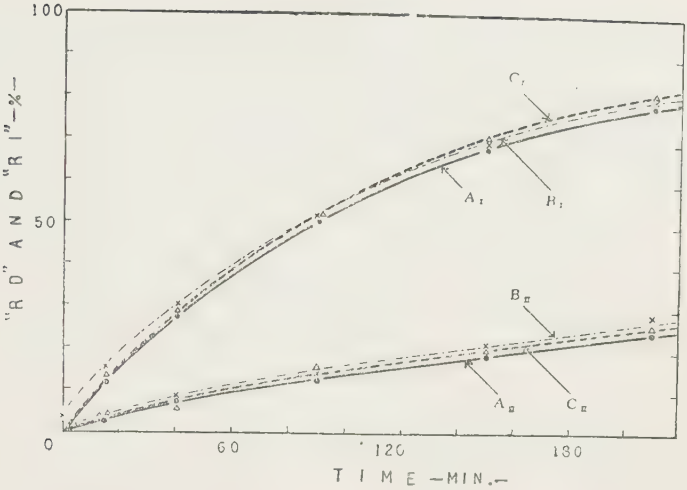

FIG. 1. Effect of calcium ion on the inactvation and denaturation of a bacterial amylase in the urea treatment.

After two solutions (I and II) containing the same concentration of BAH ($S^{10'}_{30^\circ}$/ml. 2410, about 0.3 per cent) were incubated at 40°, remaining activity after digestion and amount of product digested with the bacterial proteinase were measured and the " RI," the " RI ' after digestion and the " RD " were calculated as in the case of experiment shown in Table II.

Solution I contained 6 *M* urea and 0.01 *M* ammonium phosphate buffer solution of pH 7.0 and II contained 0.02 *M* calcium chloride in addition to above components. Curves A_I, B_I, C_I shows the course of " RI," " RI " after digestion and " RD " of the solution I respectively and A_{II}, B_{II} and C_{II} shows above ratios " of the solution II, respectively.

vation by substrates or related compounds. In the case of bacterial amylases, it is very difficult to see the protective effect of a substrate, starch, as it is immediately hydrolyzed on account of the extremely high turnover number of the enzyme. Maltose was slightly effective in protecting the amylase from heat, acid and urea inactivation, but, the decomposition product from starch (a mixture of α-limitdextrin, maltose, and a small amount of glucose) was more effective. Therefore, the effect of the decomposed starch was investigated on the inactivation and denaturation of a bacterial amylase in urea treatment. In the experiment shown in Fig. 2, one of bacterial amylases (BAH) was treated with 7.2 *M* urea at 40° and at pH 7.0 in the presence and absence of starch. As shown in the figure, both inactivation and denaturation was fairly protected by the addition of starch, though the effect is not so great as in the case of calcium ion. The above effect must be derived from the hydrolysis products of starch, probably α-limitdextrin, as starch was completely hydrolyzed within 1 minute under the experimental conditions.

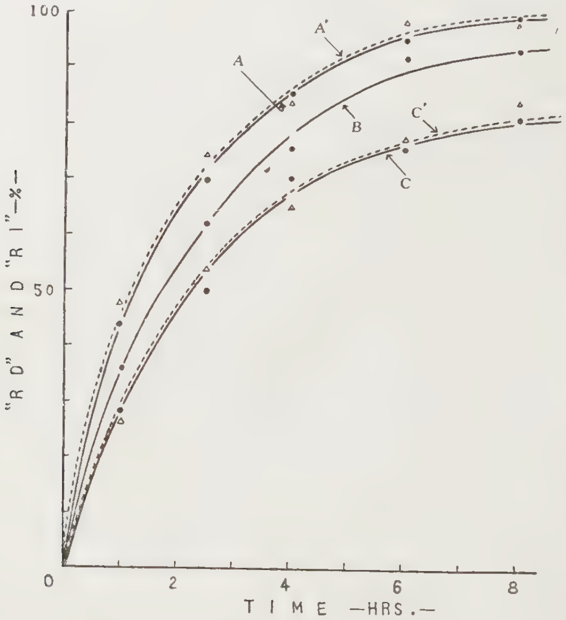

FIG. 2. Protective effect of starch digestion product on the inactivation and denaturation of a bacterial amylase in the urea treatment.

Three solutions (A, B and C) of the same concentration of BAH (about 0.15 per cent) of pH 7.0 containing 7.2 *M* urea were incubated at 40°. All solutions contained 0.01 *M* K and Na phosphate buffer. Potato soluble starch were added to the solns. B and C to the concentration of 1 and 4 per cent, respectively, before mixing with urea while it was not added to the solution A. The "RI" (from dextrinizing activity) and "RD" (by Folin method) were measured as before in the duration of the treatment.

Curves A, B and C show the "RI" of solns. A, B and C, respectively, and A′ and C′ show the "RD" of solns. A and C, respectively.

DISCUSSION

Since the " ratio of inactivation " of a crystalline bacterial α-amylase (BAN′) inactivated partially by the treatment with heat, acid, and urea agreed well with the " ratio of denaturation " of the same sample measured by the proteolysis method, it is confirmed that the inactivation of this amylase by the above treatment must arise from denaturation of the protein, *i.e.* the deformation of intramolecular structure which makes the protein susceptible to bacterial proteinase, as well as in the case of Taka-α-amylase partially inactivated with urea (*1*). As the same results were obtained when the inactivation and denaturation of another crystalline bacterial amylase (BAH) was measured at several stages in the heat or urea treatment (see Table II and Fig. 1), it may be concluded that inactivation of all kinds of α-amylase from *B. subtilis* will occur in parallel with these denaturation during heat, acid, and urea treatments. The evidences mentioned above make it quite clear that the inactivation of the bacterial α-amylases by the above treatments arises not from a special change in the essential radical or a small part of the enzyme molecule but from the alteration of intramolecular structure as a whole. It was also confirmed from the above results that the proteolytic method gives an accurate basis for the determination of the " ratio of denaturation " of the bacterial amylases, similarly as in the case of Taka-α-amylase (*1*).

It has been reported that all of typical α-amylases such as pancreas (*4*), malt (*4*, *5*), *Aspergillus* (*4*, *6*), and bacterial α-amylase (*2*, *7*), are strongly protected by calcium salts from heat inactivation. In the present investigation, it has been clearly certified that a bacterial α-amylase was highly protected by calcium ion not only from inactivation with heat but also from that with acid or urea. On the other hand, β-amylase of malt and sweet potato and saccharifying amylase of *B. subtilis* (*8*) were practically not protected by calcium ion. Since the protective effect of calcium ion on amylase is specific to α-(dextrinizing) amylases, as mentioned above, following two mechanisms may be assumed for the protective effect of calcium ion: 1) Calcium ion protects only the essential radical or a small part (the active center) of α-amylase from a change to lose their function; 2) The ion protects the whole intramolecular structure of the enzyme protein from alteration of losing the native structure (denaturation). As the results shown in Fig. 1 made it clear that the " RI " and " RD " agreed well with one another at every stage during urea treatment, both in the presence and absence of calcium ion, the second assumption must be true. Therefore, it is supposed that all kinds of α-amylase have a common or very similar portion which is comparatively labile but is highly stabilized by combination with calcium, in the very important site in the molecule for maintaining the whole structure of the molecules. The same supposition may be given to the protective effect of the digestion products from starch on the inactivation and denaturation of amylase, though its effect is compartively smaller than in the case of calcium.

SUMMARY

Inactivation and denaturation of two crystalline bacterial α-amylases (BAN′ and BAH) were investigated by assaying the enzymatic activity and determining denaturation by the proteolytic method, at the same time in the case of heat and urea treatment. The " ratio of inactivation " (RI) and the " ratio of denaturation " (RD) of BAN′ agreed well, when the amylase was partially inactivated by the foregoing three treatments. Above two ratios, RI and RD, of another bacterial amylase (BAH) also agreed well at every stage during heat and urea treatments. Therefore, it was concluded that the inactivation of all kinds of α-amylases from *B. subtilis* by the above three treatments must arise not from a special change in the essential radical (or part) of the enzyme molecule but from the alteration of intramolecular structure as a whole. Inactivation of a bacterial amylase by urea treatment was much influenced by the concentration of urea and by temperature, but it was practically uninfluenced by pH in neutral or slightly alkaline solution.

The bacterial α-amylase was strongly protected by calcium ion from inactivation and denaturation by heat, acid, and urea treatments as in the case of other α-amylases. Since the " RI " and " RD " of the amylase agreed well at every stage in the urea treatment, even in a solution protected by the presence of calcium ion, it was supposed that the protective effect of calcium ion on all α-amylases is derived from the protection of a common (or very similar) part in the molecule of all α-amylases which is very important in maintaining the whole secondary structure of the molecule. The bacterial amylase was also protected by the presence of digestion products of starch, from both inactivation and denaturation to the same degree during urea treatment, though the magnitude of the protection was not large.

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DENATURATION AND INACTIVATION OF ENZYME PROTEINS

III. DENATURATION AND INACTIVATION OF TAKA-α-AMYLASE

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It has been reported (*1*, *2*, *3*) that the "ratio of denaturation" of some enzyme proteins denatured partially by various treatments was measured accurately by a very simple method using proteolysis with crystalline bacterial proteinases. Measuring the magnitude of the denaturation by this method it has been ascertained (*4*) that the "ratio of inactivation" and the "ratio of denaturation" of crystalline α-amylases of *B. subtilis* coincide well with each other at every time in the duration of heat and urea treatment. Furthermore, it has been concluded from the results obtained with the bacterial α-amylase that the protective effect of the calcium ion or the digestion product of starch on the inactivation of α-amylases was not attributed to protection of the active radical from a specific change but to protection of the secondary intramolecular structure of the proteins from alteration (denaturation) as a whole. In the present investigation, inactivation and denaturation of crystalline Taka-α-amylase were studied by assaying the enzymatic activity and determining the denaturation by the described proteolytic method at the same time in the duration of heat, urea and acid treatment. Though some of the results of the above investigation have been briefly reported elsewhere (*2*, *3*), the purpose of this report is to present more detailed evidence, especially that for the reversible denaturation and inactivation of Taka-α-amylase.

MATERIALS AND METHODS

Crystalline Taka-α-amylase—Crystallized from Taka-diastase Sankyo (a preparation obtained from a wheet bran culture of *Aspergillus oryzae*) according to the method of Akabori, Ikenaka and Hagihara (*5*) and recrystallized 3 times from acetone solution containing 0.01 *M* calcium acetate.

Crystalline Bacterial Proteinase—BPR, proteinase of *B. subtilis* R (*2*), was used (*1*).

Assay for Amylase Activity—The activity was measured mainly by the starch-dextrinizing power using a standard colour solution as the end point and the unit was represented by $D^{30'}_{40°}$ (*2*). But it was measured by the saccharifying power when a more exact result was required (see the 1st (*1*) and 2nd reports (*4*)).

Method for Assaying the " Ratio of Denaturation "—The main procedures were as in the 2nd report (*4*), but they were slightly modified as described in the each section of the results.

Other procedures will be described in the appropiate section.

RESULTS

Experiment 1

Inactivation and Denaturation in the Duration of Heat Treatment

About 0.5 per cent solution of crystalline Taka-α-amylase containing virtually no salt was treated at 60°, and the remaining activity and the magnitude of denaturation of the amylase protein were occasionally measured in order to make clear the course of inactivation and denaturation of the amylase and also to see whether the " ratio of inacivation " (RI) and the " ratio of denaturation " (RD) coincide with one another or not at every stage in the duration of the heat treatment. The results are shown in Table I. As supposed from the result obtained in the case of a partially denatured Taka-α-amylase with urea (*1*) and the evidence obtained in the duration of heat treatment of a bacterial α-amylase (*4*), the " RI " and the " RD " of Taka-α-amylase agreed well with one another at every stage in the described heat treatment. Since the reaction rate constant (k_1) of inactivation and denaturation, calculated as first order reactions, were very near values to each other during the treatment, it was concluded that both reaction proceeded as unimolecular reactions, which was also indicated in the earlier part, though not throughout the whole process, of the course of the bacterial α-amylase by heat treatment (*4*).

Experiment 2

Inactivation and Denaturation in Urea Treatment in the Absence and Presence of Calcium Ion

When about 0.6 per cent Taka-α-amylase solution dissolved in distilled water was treated with 6 *M* urea at 40°, both inacivation and de-

TABLE I

Inactivation and Denaturation of Taka-α-amylase in the Duration of Heat-treatment

Time of treat. (*min.*)	Activity test			Proteolysis		
	Remaining ($D^{30'}_{40^\circ}$)	"RI" (%)	Rate-const. (k_1)	Folin color (1/14)* (OD_{test})	"RD" (%)	Rate const. (k_1)
0	4600	0		0.028**	0	
15	4250	7.6	0.0023	0.076	11.2	0.0034
30	3620	21.5	35	0.124	21.2	35
45	3020	34.4	40	0.163	32.6	38
60	2780	39.6	37	0.196	40.8	38
80	2260	50.9	38	0.234	49.8	37
100	1820	60.5	40	0.270	58.4	38
120	1580	65.7	38	0.296	64.8	38
150	1280	72.2	37	0.320	70.5	36
Completely denatured	0	100		0.442***	100	

Crystalline Taka-α-amylase was dissolved in distilled water and pH of the solution was adjusted to 6.5 with small amount of ammonia. The solution (about 0.5 per cent, $D^{30'}_{40^\circ}$/ml.=4,600) was kept at 60°. By denaturation in the above condition, the amylase was not precipitated at all. At each time in the duration of the treatment, 2 test solutions of the same volume (1 ml.) were pipetted from above solution at the same time and one was immediately diluted with 0.01 *M* Na_2HPO_4 solution (500 ml.) for assaying amylase activity. The another solution was mixed with about 0.05 per cent BPR solution containing 0.1 *M* phosphate buffer of pH 7.5 (2 ml.) and incubated at 40° for 10 minutes. To the above digested mixture, 1 *M* TCA (2 ml.) was added and the concentration of digestion product soluble in 0.4 *M* TCA was measured by Folin's method (1, 4). The optical density of the Folin colored solution, by mixing 1 ml. of the filtrate with 5 ml. of Na_2CO_3 and 1 ml. of 1/5 Folin reagent, was measured at 660 mμ in 0.5 cm. layer (*). The Folin colore value at 0 time (**) was used as $OD_{nat.}$ and the value for the sample completely denatured at 80° for 1 hr. (***) was used as $OD_{den.}$ in the calculation by the following equation:

$$RD\ (\%) = \frac{OD_{test} - OD_{nat.}}{OD_{den.} - OD_{nat.}} \times 100$$

naturation proceeded almost in parallel with each other according to the unimolecular reaction and the "RI" and the "RD" well coincided with one another at every stage in the treatment as shown in Table II.

TABLE II

Inactivation and Denaturation of Taka-α-amylase in the Duration of Urea-treatment in the Absence and Presence of Calcium Ion

Time of treat. (*min.*)	Absence or presence of *M*/30 $CaCl_2$	Activity test			Ploteolysis		
		Remaining ($D^{30'}_{40^\circ}$)	"RI" (%)	Rate. const. (k_1)	Folin colore (1/14) (OD_{test})	"RD" (%)	Rate const. (k_1)
0 (native)	−	5200	0		0.040	0	
	+	5200				0	
10	−	4800	7.7	0.0035	0.088	10.4	0.0048
	+	5100	1.9	0.00083	0.047	1.5	0.00066
20	−	4000	23.0	0.0056	0.143	22.1	0.0054
	+	5100	1.9	0.00042	0.051	2.4	0.00053
40	−	3300	31.5	0.0041	0·214	37.3	0.0051
	+	4900	5.8	0.00066	0.069	6.2	0.00070
60	−	2700	48.0	0.0047	0.278	51.0	0.0052
	+	4600	11.5	0.00088	0.085	9.7	0.00074
100	−	1500	63.5	0.0044	0.352	66.8	0.0048
	+	4300	17.0	0.00081	0.115	16.1	0.00076
150	−	1250	76.0	0.0041	0.403	77.8	0.0044
	+	4000	23.0	0.00076	0.144	22.3	0.00073
220	−	770	85.0	0.0037	0.432	84.0	0.0036
	+	3600	31.0	0.00073	0.187	31.5	0.00075
Completely denatured		0	100		0.466	100	
		0	100			100	

Crystalline Taka-α-amylase was dissolved in distilled water and pH of the solution was adjusted to 8.0 with ammonia. The above solution containing about 1.2 per cent amylase ($D^{30'}_{40^\circ}$/ml. = 10,400) was mixed with the same volume of 1.2 *M* urea solution of pH 8.0 and the resulting solution (about 0.6 per cent, $D^{30'}_{40^\circ}$/ml. = 5,200) was incubated at 40°. Inactivation and denaturation of the amylase in the duration of above treatment were measured as in the case of *Expt. 1* (see Table I). As a test solution for zero time treatment, an aliquot of the above solution was used immediately after the mixing. For the experiment in the presence of calcium ion, the enzyme solution was mixed with the urea solution containing *M*/15 $CaCl_2$ as above.

When the test solution containing calcium ion was treated as above Taka-α-amylase was strongly protected from both inactivation and denaturation to the same degree as in the case of α-amylase of *B. subtilis*. Taka-α-amylase was also protected in the presence of calcium ion from both inactivation and denaturation by heat or acid treatment.

Experiment 3

Inactivation and Denaturation in the Duration of Acid Treatment

Though Taka-α-amylase is very stable in the solution of pH 4~12 at room temperature (*6*), it was inactivated gradually at about pH 3.0 and rapidly below pH 2.0. When about 0.5 per cent solution of crystalline Taka-α-amylase containing 0.01 *M* phosphoric acid (about pH 2.8) was kept at 30°, denaturation of the amylase measured by the proteolytic method proceeded with time as shown by Curve A in Fig. 1. However, inactivation of the amylase was always smaller than the denaturation at every time in the duration of the above acid treatment, if the activity was measured after each test solution was diluted with 0.005 *M* sodium monohydrogen phosphate solution. Since the difference between the " RD " and " RI " at every time in the treatment was more pronounced when the activity was measured at 24 hours after the dilution (Curve C) than at 1 hour (Curve B), it was supposed that the amylase inactivated by the acid treatment was reactivated in the period before assaying, *viz*. after the dilution by which the pH of the test solution was neutralized. As S. Akabori *et al*. (*7*, *8*) has already pointed out Taka-α-amylase inactivated by acid treatment is gradually reactivated after neutralization. It was clear from Curve A in Fig. 1 that the amylase inactivated to the reversible state was also susceptible to the proteolytic digestion. Therefore, it was supposed that the true activity of the test solutions at every instance in the duration of the acid treatment could be measured when the test solutions were diluted after incubation with proteinase in order to digest the reversibly inactivated amylase. As shown by Curve D in the Fig. 1, the " RI " of the amylase measured as above well coincided with the "RD" shown by Curve A, as in the cases of heat or urea treatment, in which the reactivation of the inactivated amylase did not occur.

Experiment 4

Inactivation and Denaturation in the Process of Reactivation of Taka-α-amylase Inactivated with Acid

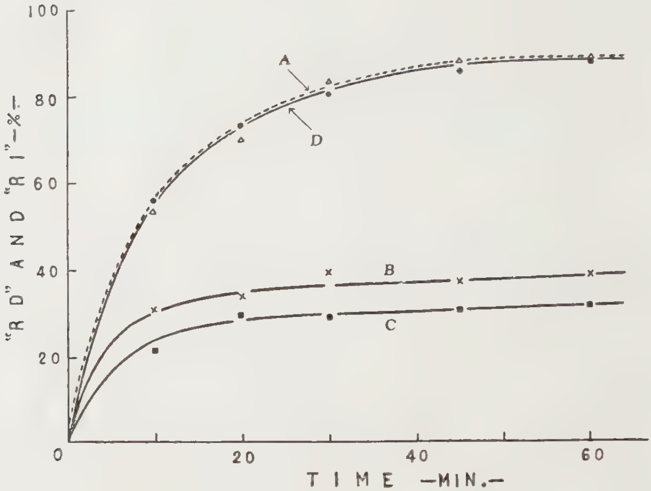

Fig. 1. Courses of inactivation and denaturation of Taka-α-amylase in the duration of acid-treatment.

About 1 per cent crystalline Taka-α-amylase solution in distilled water was mixed with the same volume of 0.02 *M* H_3PO_4 and resulting mixture of about pH 2.8 was kept at 30°. At each time in the duration of the treatment, 2 test solutions, one of which was 0.5 ml. and another was 1 ml. were pipetted from above solution at the same time. The first solution (0.5 ml.) was immediately diluted to 300 ml. (1/600) with 0.005 *M* Na_2HPO_4 solution containing 0.02 *M* NaCl and amylase activity of the diluted solution was measured by the starch saccharifying power at 1 and 24 hours after the dilution. The second solution (1 ml.) was immediately mixed with 2 ml. of 0.1 per cent BPR solution containing 0.1 *M* Na_2HPO_4 (1/3) and the mixture (about pH 7.5) was kept at 40° for 10 minutes. From above digested mixture, 0.5 ml. was pipetted and diluted to 100 ml. (end 1/600) for the assay of amylase activity and 2 ml. was mixed with 2 ml. of 0.8 *M* TCA for determining the ratio of denaturation which was carried out as before.

A ······△······ Ratio of denaturation by the proteolytic method
B ——×—— Ratio of inactivation measured 1 hour after dilution
C ——■—— Ratio of inactivation measured 24 hours after dilution
D ——●—— Ratio of inactivation measured after the proteolysis

When a solution of Taka-α-amylase inactivated partially by urea treatment was kept at room temperature for a fairly long time after dilution with a neutral buffer solution, or after dialysis against the same solution, by means of which the residual urea was respectively either diluted or removed, the activity of the amylase was not increased at all with time of the keeping. From above result and results of *Expt. 2* in which the " RD " and the " RI " measured after dilution coincided well with one another, it was concluded that the amylase inactivated by urea could not be reactivated by removing urea. Similarly as above, Taka-α-amylase inactivated once by heat treatment in neutral or weakly acidic solutions was not practically reactivated by cooling alone. On the contrary, Taka-α-amylase inactivated by acid treatment was readily reactivated by neutralization as already reported by Akabori *et al.* (*7, 8*).

In the present investigation, about 0.5 per cent Taka-α-amylase solution treated with acid by the same conditions as *Expt. 3* for 60 minutes (about 85 per cent inactivation) was neutralized to pH 7.0 with sodium hydroxide and kept at 30°. As shown by Curves A and C in Fig. 2, the " RD " of the amylase rapidly decreased with time in above incubation in parallel with the " RI " measured after the proteolysis, though both ratios were slightly different from one another at the earlier period of the incubation. This difference was supposed to be attributable to experimental error. However, as shown by Curve B the " RI " measured at 1 hour after the dilution without proteolysis were considerably different from the " RD " at the same time in the reactivation process, especially in the earlier period, since the inactivated amylase contained in the solution could be further reactivated by the dilution.

DISCUSSION

About the Proteolytic Method for the Determination of Denaturation—It has been already reported in the previous paper (*4*) that the " ratio of denaturation " measured by the proteolytic method (*1*) well coincided with the " ratio of inactivation " at every stage in the duration of heat and urea treatment of a crystalline bacterial α-amylase. Since the bacterial α-amylase produced by *B. subtilis* together with the proteinase which was being used in the proteolytic method and both enzymes were contained for a long time in the same solution before they were separated by the purification procedures, it was readily supposed that the amylase was very resistant to the proteolysis by above proteinase. This fact might be one important reason for the fact that " RD " of the bacterial α-amylase could be measured quite accurately by the proteolytic method at every stage of the denaturation. However, a brief result has been

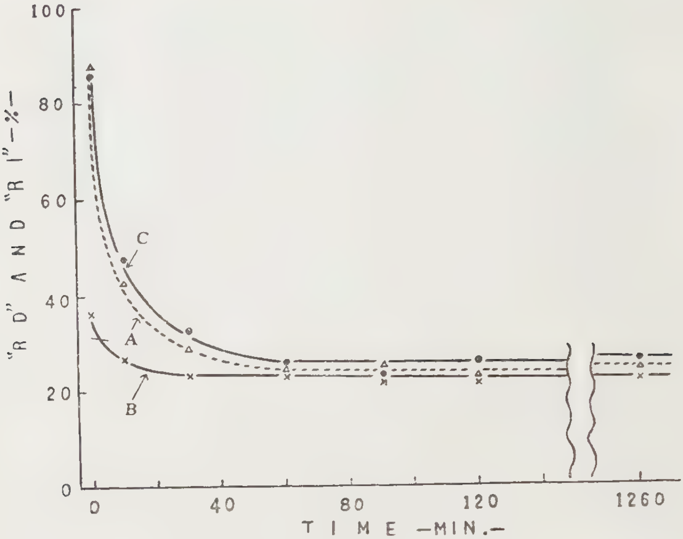

FIG. 2. Course of reactivation and renaturation of acid-denatured Taka-α-amylase after neutralization.

About 0.5 per cent solution of Taka-α-amylase denatured with acid to about 86 per cent inactivation at the same condition as *Expt. 3* (see description under Fig. 1) was neutralized to pH 7.0 by addition of the same volume of 3/200 *N* NaOH and kept at 30°. At each time in the duration of above incubation, 2 test solutions 1 ml. and 2 ml., were pipetted out from above solution at the same time. The first solution (1 ml.) was immediately diluted to 300 ml. and the activity was measured 1 hour after the dilution. The another (2 ml.) was immediately mixed with 1 ml. of 0.2 per cent BPR solution containing 0.2 *M* phosphate buffer of pH 7.5 and treated similarly as *Expt. 3*.

A ······△······ Ratio of denaturation by the proteolytic method
B ——×—— Ratio of inactivation measured 1 hour after dilution
C ——●—— Ratio of inactivation measured after the proteolysis

already reported in the first paper (*1*) which showed the coincidence of the " RI " and the " RD " measured with a bacterial proteinase in the case of a partially denatured Taka-α-amylase with urea, and furthermore, it was shown in the present investigation that above both ratios well coincided with one another at every stage in the duration of heat and urea treatment of Taka-α-amylase. Therefore, it was further confirmed that the proteolytic method is also a very accurate method for determining the " RD " of proteins other than bacterial α-amylase.

In the case of acid treatment of Taka-α-amylase, the " RD " did not always agree with the " RI " measured by the ordinary procedure. But, it was concluded that the " RD " showed the true value at the instant of sampling in the treatment and the " RI " did not show the true value at the instant, on account of the reactivation as discussed later. Therefore, the proteolytic method is thought to be useful for the determination of the " RD " of proteins denatured even in the reversible state (see the 4th section).

Time Course of Denaturation—It has been reported that denaturation of bacterial α-amylase proceeds according to the unimolecular reaction in the earlier period in the duration of heat and urea treatment (*4*). As shown in Tables I and II of the present paper, Taka-α-amylase was denatured good in accordance with a unimolecular reaction, until a later period in the treatments than in the case of bacterial α-amylase. However, it cannot be concluded from above few results that the time course of denaturation of Taka-α-amylase falls in always more favourably with the unimolecular reaction than that of bacterial α-amylase, since both amylases were treated under conditions differing in detail.

Protective Effect of Calcium Ion—Reasons have been given in the previous paper (*4*) for believing that the protective effect of calcium ion on the inactivation of bacterial α-amylases was not caused by the protection of an active radical from a special change, but by the protection of the secondary intramolecular structure of the enzyme protein from alteration (denaturation) as a whole, because this amylase was protected from both inactivation and denaturation to the same degree in the presence of calcium ion. In the present investigation, it was confirmed that the " RI " and " RD " of Taka-α-amylase also well coincided with one another at every stage in the duration of urea treatment irrespective of the presence or absence of calcium ion as in the case of bacterial α-amylase. Therefore, the above conclusion, that the calcium ion is a protector of denaturation, seems to be more favourable.

Reversible Denaturation—When bacterial α-amylases (*B. subtilis*) were once inactivated by heat, urea or acid treatment, the inactivated amylases were not reactivated by any way tested such as cooling, dialysis, dilution or neutralization. Taka-α-amylase inactivated by urea treatment was not also reactivated. When Taka-α-amylase was inactivated by heating about at 70° in neutral or slightly alkaline solutions the amylase was not reactivated to a measurable degree. However, the amylase inactivated by similar heating in a slightly acidic solution was reactivated to some degree by neutralization and cooling. As Akabori *et al.* (7, *8*) has already reported, the amylase inactivated in fairly acidic solution was strongly reactivated by keeping at cold after neutralization, although it was not possible to reactivate completely all of the inactivated amylase. It was supposed that Taka-α-amylase was denatured by acid treatment to the reversible state because some parts of the molecule was protected by disulfide crosslinkage (about 4 disulfide bonds have been reported to be present in a molecule of Taka-α-amylase by Akabori *et al.* (*9*)) and bacterial α-amylase was denatured irreversibly by the same treatment because this amylase has no disulfide bond (*10*).

When a test solution was pipetted from a Taka-α-amylase solution at a given instance in the duration of acid treatment and diluted with a neutral or weakly alkaline buffer solution for the assaying activity, a considerable amount of denatured amylase contained in the test solution was reactivated in the period before the assaying, even if the procedure was carried out as rapidly as possible. When the test solution was diluted with distilled water, reactivation also occurred though the extent of reactivation was smaller than the above case. If the solution is diluted with an acidic solution, the reactivation may be prevented, but further denaturation of the test sample may occur. Even in the case when the amylase is treated with acid in a very diluted condition and the activity is measured without dilution—though the concentration of amylase is too small to measure the " RD " at the same time—, some part of the denatured amylase may be reactivated during the assaying procedure, which is carried out in the solution of almost neutral pH. Therefore, it is very difficult to measure the true activity of an enzyme at a given instant in the duration of denaturation procedure by which the enzyme is reversibly denatured as above.

If the active (native) molecules of the amylase are not digested at all by the proteolysis and all of the inactivated molecules including reversibly inactivated ones are completely digested by it, the " RD " of the amylase measured by the proteolytic method is supposed to coincide with the " RI," measured after the proteolysis, by which the reactivatable molecules are removed. The results of *Expt. 3* showed that the above supposition is applicable to every stage in the duration of acid treatment of Taka-α-amylase. Though it is supposed that the reversibly denatured amylase included in the test solution may be reactivated (renatured) in a very short period which corresponds to the time till the reversibly denatured amylase is digested to the state unable to be renatured after the test solution is mixed with the proteinase solution, above reactivation is thought to be negligibly small, since the reactivation is a time requiring reaction and the amylase contacts with a considerably concentrated proteinase at a instance of neutralization. From above consideration, it seems to be a very convenient method to measure the activity after incubation with the proteinase for the determination of true activity of enzyme proteins at a given instance both in the course reversible denaturation and in the course of renaturation.

Using the technique mentioned above and ordinary assaying procedure, the course of reactivation of a acid-denatured Taka-α-amylase was investigated together with the course of renaturation of the protein in *Expt. 4*. As the " RI " measured after the proteolysis was almost the same as the " RD " (see Fig. 2), it was concluded that the reactivated Taka-α-amylase protein has similer resistance to the bacterial proteinase as the intact native Taka-α-amylase. With regard to the reactivation process of Taka-α-amylase, more detailed investigation is in progress in this laboratory.

SUMMARY

Inactivation and denaturation of crystalline Taka-α-amylase were investigated in the case of heat, urea and acid treatment. The " ratio of inactivation " (RI) and the " ratio of denaturation " (RD) of the amylase well coincided with one another at every stage in the duration of heat and urea treatment, and the time course of denaturation (inactivation) of the amylase in the above treatment proceeded almost in accordance with the unimolecular reaction. Protective effect of calcium ion on the inactivation and denaturation of the amylase was the same as in the case of bacterial amylase (*4*). When the amylase was treated in an acidic solution, the amylase was reversibly denatured and, therefore, the " RD " did not coincided with the " RI " measured by the ordinary procedure, on account of the reactivation in the assaying procedure. But, when the activity was measured after the proteolysis, by which the reactivatable molecules were removed, the " RD " and the " RI " well coincided with one another at every stage in the course of acid denaturation. The same result as above was obtained in the process of reactivation of acid denatured Taka-α-amylase. Therefore, the proteolytic method for determining the " RD " of the partially denatured

protein is confirmed to be useful even in the reversible denaturation and renaturation process.

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DENATURATION AND INACTIVATION OF ENZYME PROTEINS

IV. DENATURATION AND INACTIVATION OF CATALASE

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Using the proteolytic method (*1*) for the determination of the "ratio of denaturation" of partially denatured enzyme proteins, it has been confirmed that the inactivation of some crystalline enzymes, such as α-amylases of *Aspergillus oryzae* and *Bacillus subtilis*, proceeds in parallel with the denaturation of the protein in the duration of urea-, heat- and acid-treatment (*2*, *3*). In the previous paper (*3*), it has been clearly established that *Aspergillus* (Taka)-α-amylase, denatured to the reversible state by an acid-treatment, was perfectly susceptible to the bacterial proteinase and the "ratio of denaturation" of the protein even in the reversibly denatured state could be measured as in the case of the irreversibly denatured proteins.

The amylases used in the previous reports (*1*, *2*, *3*) were very stable enzymes and thought to be composed of simple protein. In the present investigation, crystalline beef catalase which is comparatively less-stable and one of the conjugated proteins was used in order to confirm whether the "ratio of denaturation" of this protein could be determined accurately by the proteolytic method or not when the protein was treated with urea as a typical denaturing agent or benzoate as a perturbator (*8*, *9*). Furthermore, the relation between denaturation and inactivation of the catalase was also investigated, when the enzyme was inhibited by the specific inhibitors such as cyanide, fluoride, azide, or hydroxylamine. Though many results have been reported as to the inhibition of catalase activity in the presence of the above reagents, it has never been ascertained whether these inactivations resulted from the denaturation of the enzyme protein with specific inhibitors or from a simple combination of the inhibitors at the essential radicals of the enzyme without alteration of the whole intramolecular structure—denaturation. Though some results of the above investigation have been reported briefly elsewhere (*4*, *5*), more detailed experiments will be described showing that the specific inhibitors of heme protein affect only the activity of enzyme, but do not denature the enzyme protein.

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MATERIALS

Crystalline Catalase—The enzyme was prepared from beef liver according to the slightly modified method of Kitagawa and Shirakawa (*6*) and recrystallized four times.

The stock solution of the catalase was prepared dissolving the crystals with 0.02 M Na_2HPO_4 and the pH of the solution was adjusted to 6.8 with 0.02 M KH_2PO_4. Approximately 0.4 or 0.2 per cent solution, prepared as above, was used rapidly for the treatment with urea or the specific inhibitors, respectively.

Denaturing Agents and Specific Inhibitors—Urea was used as a typical denaturing agent. To make clear the mechanism of action of so-called "perturbators" (*8*) against heme proteins, sodium benzoate and sodium salicylate were used. As specific inhibitors, potassium cyanide, sodium fluoride, sodium azide, and hydroxylamine were used. These reagents were all commercial preparations.

All of the above agents were dissolved in 0.02 M phosphate buffer solution and pH of the solutions were adjusted to 6.8.

Crystalline Bacterial Proteinase—Though BPR (proteinase of *Bac. subtilis R* (*5*)) was mainly used in the previous reports (*2*, *3*), BPN′ (proteinase of *Bac. subtilis N*) was used in the present investigation since it has become easily obtainable as a commercial preparation*. As a proteinase for the determination of the " ratio of denaturation" of proteins, BPN′ is equal as usefully as BPR (*1*).

METHODS

Assay for Catalase Activity—When a test solution of the catalase was diluted with distilled water, the catalase was considerably inactivated even at cold. But the catalase was not inactivated, if the test solution was diluted with ice cold 0.02 M phosphate buffer solution of pH 6.8 containing 0.05 M sodium chloride. The activity of the catalase was measured according to the method of Euler and Josephson (*7*) as follows:

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* Crystalline proteinase of *Bac. subtilis* has been prepared in a large scale under the authors' guidance at Nagase Ltd. (Amagasaki, Japan), and is now easily obtainable as a commercial preparation " Nagarse."

1.0 ml. of the diluted enzyme solution was pipetted into 50 ml. of 0.005 *N* hydrogen peroxide solution containing 0.1 *M* phosphate buffer of pH 6.8 maintained at 0°, and each 5 ml. of samples were pipetted from the reaction mixture into 2 ml. of 2 *N* sulfuric acid at the 1st, 4th, 7th, and 10th minute from the begining of the reaction. The remaining hydrogen peroxide in the above test solutions was titrated with 0.005 *N* potassium permanganate and the unimolecular reaction constants (k) were calculated, then the value at zero time was obtained by extrapolation. Since the relation between enzyme concentration and above k value at zero time showed a slight deviation from the linear relation if a wide rage of the enzyme concentration was tested, the value was corrected by a standard curve in order to show the one in proportion to concentration of active enzyme.

Determination of the "Ratio of Denaturation"—The main procedures were carried out similary to those described in the previous reports (*1*, *2*, *3*), but they were slightly modified in detail as follows: 1 ml. of the catalase solution (test solution) was added to 2 ml. of about 0.05 per cent solution of the bacterial proteinase containing 0.1 *M* phosphate buffer of pH 7.5 and incubated at 30° for 10 minutes in the case of urea-treatment or at 20° for 15 minutes in other cases. After the above incubation, 1 ml. of 1.6 *M* trichloroacetic acid (TCA) was added and the concentration of the digestion product soluble in 0.4 *M* TCA was measured by Folin's colour reaction as before (*1*, *2*). In order to determine the value of a completely denatured sample, the same aliquot of the test solution was denatured by heating at 70° for 10 minutes and treated with the proteinase solution as above. The "ratio of denaturation" (RD) of catalase protein in a test solution was calculated as follows (*1*):

$$\mathrm{RD}\ (\%)=\frac{\mathrm{OD}_{\mathrm{test}}-\mathrm{OD}_{\mathrm{native}}}{\mathrm{OD}_{\mathrm{denat.}}-\mathrm{OD}_{\mathrm{native}}}\times 100$$

Treatments with Various Agents—These procedures will be described in the each section of the results.

RESULTS

Experiment 1

Inactivation and Denaturation in Urea-treatment

When about 0.2 per cent catalase solution of pH 6.8 containing 3.5 *M* urea was incubated at 30° the "ratio of inactivation" (RI) and the "ratio of denaturation" (RD) almost coincided with one another at every stage in the duration, but the latter values were slightly higher as shown in Table I and Fig. 1. If the activities were measured after the proteolysis, the "RI" coincided well with the "RD" measured at the same time (see Curve B and C in Fig. 1). It was supposed that the small difference between the "RI" measured without proteolysis (Curve A) and the "RD" (Curve C) might be due to either reactivation

TABLE I

Inactivation and the Proteolytic Susceptibility of Crystalline Beef Catalase in the Duration of Urea-treatment

Time of treat.	Proteolysis	Activity test				Proteolysis	
		Activity ($k_1 \times 1/4000$)		"Ratio of inact."(%)		Folin-Colour (OD_{test})	"RD" (%)
		After dilution		After dilution			
min.		Immediate	24 hrs.	Immediate	24 hrs.		
0	−	0.100	0.100	0.0	0	($OD_{nat.}$)	0.0
	+	0.099	0.097	1.0	3	0.025	
20	−	0.093	0.096	8.0	3.5	0.056	9.5
50	−	0.084	0.083	16.3	17.2		
	+	0.080	0.072	20	27.9	0.085	18.7
80	−	0.080	0.081	20	19.3	0.106	24.7
120	−	0.075	0.074	25	26.0		
	+	0.070	0.061	30	38.9	0.124	30.3
210	−	0.066	0.065	34	34.9		
	+	0.061	0.052	39	47.7	0.157	40.4
390	−	0.052	0.051	48	48.6		
	+	0.045	0.035	55	65.4	0.211	56.8
Complete denaturation	−	0		100		($OD_{den.}$)	
	+					0.352	100

About 0.4 per cent catalase solution containing phosphate buffer of pH 6.8 was mixed with the same volume of 7.0 *M* urea solution and the mixture was incubated at 30°. At each time interval in the duration of the incubation, 3 test solutions, 0.5 ml., 1 ml. and 1 ml., were pipetted from the above solution and the 1st 0.5 ml. was immediately diluted to 1/4100 with the cold salts solution by two steps in order to assay the catalase activity. The 2nd and 3rd test solutions were immediately mixed with 2 ml. of the proteinase solution, and incubated at 30° for 15 minutes. One of above digested solutions was diluted to the same degree as the 1st one in order to assay "the activity after the proteolysis" and the other solution was used for determination of the "ratio of denaturation."

of inactivated catalase by the dilution as in the case of acid-treatment of Taka-α-amylase (*3*) or slight digestion of the native catalase with proteinase. Therefore, the activity of the diluted solutions was measured after 24 hours to see whether further reactivation or further loss in avtivity would occur. When the samples were diluted without proteolysis and kept at 10° for 24 hours, the avtivities (Curve A′) were not appreciably different from the corresponding activities measured immediately after dilution (Curve A). Therefore, it was difficult to conclude that the catalase inactivated once with above urea-treatment had been reactivated by dilution as in the case of Taka-α-amylase inactivated with acid-treatment. On the contrary, if the samples were diluted after mixing with the proteinase and kept at 10° for 24 hours, the activities (Curve B′) were much lower than the corresponding activities measured immediately after dilution (Curve B). This result showed that even an active molecule of catalase is slightly susceptible to the proteinase, though it is much more resistant than a denatured molecule of catalase.

Experiment 2

Action of Perturbators against Catalase

It has been reported that the absortion spectrum of hemoglobin was reversibly shifted by the action of some reagents such as benzoates or salicylates which were called " perturbators " by Holden *et al.* (*8*). T. Tomimura *et al.* (*9*) have also reported that these reagents somewhat affect the spectrum, catalatic and peroxidatic activities of horse liver catalase.

In the present investigation the " RI " and the " RD " of beef catalase were measured at the same time in the duration of teratment with 1.8 *M* benzoate at 20°. As shown by the results given in Fig. 2, the inactivation and denaturation proceeded similarly as in the case of urea-treatment. Therefore, benzoate is concluded to act against catalase as a denaturing agent under this experimental concentration.

The action of sodium salicylate was also investigated, but the " RD " could not be measured by the ordinary procedure since salicylate gave a strong colour reaction with Folin reagent and also showed strong extinction at 278 mμ. Therefore, the " RD " was calculated from the amount of precipitatable protein with 0.4 *M* TCA after the proteolysis —corresponding to remaining native protein—which was rather more difficult to measure accurately than in the case of a digestion-product in the ordinary procedure. However, it was confirmed that salicylate behaved in an almost identical way to benzoate.

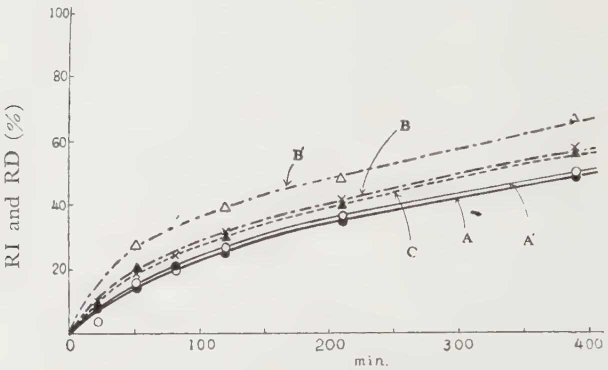

FIG. 1. Courses of inactivation and denaturation of catalase in the duration of urea-treatment.

Experimental condition ; see Table I.

A Ratio of inactivation measured immediately after dilution.
A′ ,, ,, measured 24 hrs. after dilution.
B ,, ,, immediately after dilution following the proteolysis.
B′ ,, ,, 24 hrs. after dilution following the proteolysis.
C Ratio of denaturation measured by the proteolysis method.

Experiment 3

Action of Cyanide

As it is a well known fact that the activity of catalase is strongly inhibited by cyanide at the final concentration of 10^{-6} *M* in the solution of enzyme reaction, the relation between inactivation and denaturation of catalase was studied in the treatment with 10^{-2} *M* potassium cyanide. In the experiment, concentration of catalase was about 0.1 per cent which was the minimum concentration in order to determine the " RD " by the proteolytic method and the catalytic activity of the treated solution was measured after dilution about 2000 times for it is too strong to estimate the accurate activity. As the above diluted solution was further diluted 51 times with the substrate solution, the concentration of the inhibitor in the treated solutions was diluted about 10^5 times in the solution of enzyme reaction. On the other hand, the treated solution was diluted 3 times in the solution for determination of the " RD," since 1 ml. of the solution was mixed with 2 ml. of the proteinase solution. Therefore, concentration of the cyanide was 0.33×10^{-2} and $0.33 \times$

10^{-6} M in the solutions for determination of denaturation and about 10^{-7} and 10^{-11} M in the solutions for assay of catalase avtivity, though it was 10^{-2} M and 10^{-6} M respectively in the treated solution. The results of the experiment are shown in Table II. As shown in the Table, both denaturation and inactivation were not detected at any stage in the duration of the treatment with the cyanide even in the concentration of 10^{-2} M. Since the concentration of cyanide was sufficient to inhibit the enzyme in the treated solution, it was concluded that the enzyme was reactivated completely by dilution of the inhibitor in the process for assay of activity. In the process for determination of denaturation, the concentration of the inhibitor was also sufficient to inhibit the activity at least in the case using 10^{-2} M cyanide in the treated solution. Therefore, it was concluded from the results shown in the Table that cyanide inhibits the activity of catalase without denaturing the enzyme protein. Furthermore, it was shown from the results that the action of cyanide on catalase was not affected during the treatment at 30°.

Experiment 4

Action of Fluoride

The experiment was carried out as in the case of *Experiment 3*, except that sodium fluoride at a final concentration of 1×10^{-1} M was used. This concentration was quite sufficient to inactivate catalase completely. As shown in Table III, the same results were obtained as in the case of cyanide treatment.

Experiment 5

Action of Azide

The experiment was carried out as in the case of *Experiment 3*, except that sodium azide at a final concentration of 0.5×10^{-3} or 0.5×10^{-7} M was used. The former concentration of the azide was quite sufficient to inactivate catalase and even the latter concentration was believed to be sufficient to inactivate more or less completely. The results are shown in Fig 3. As shown in Fig. 3 the catalse was not denatured at all with this reagent even at the concentration of 0.5×10^{-3} M, similarly as in the case of cyanide or fluoride-treatment. However, considerably strong inactivation was observed even after dilution. Therefore, it seems that the catalase was inactivated by azide-treatment without denaturing the enzyme protein and the inactivated enzyme cannot be reactivated by dilution of the reagent.

TABLE II

Inactivation and Denaturation of Catalase in the Duration of Treatment with Potassium Cyanide

Conc. of of CN (*M*) / Time of treat. (*min.*)	0			10^{-6}			10^{-2}		
	Activity** (k)	OD_{test}	OD_{test}*** $-OD_{nat.}$	Activity (k)	OD_{test}*	OD_{test}** $-OD_{nat.}$	Activity (k)	OD_{test}*	OD_{test}** $-OD_{nat.}$
0	0.029	0.073	0	0.029	0.073	0	0.030	0.074	0.001
15				0.028	0.065	−0.008	0.029	0.080	0.007
30				0.028	0.060	0.003	0.026	0.070	−0.003
50				0.028	0.076	0.003	0.029	0.065	−0.008
80				0.028	0.075	0.002	0.029	0.066	−0.007
120	0.029	0.070	−0.003	0.029	0.076	0.003	0.030	0.070	−0.003
Complete denaturation	0	0.234	0.161	0	0.236	0.163	0	0.241	0.168

About 0.2 per cent solution of crystalline beef catalase was mixed with the same volume of buffer solution of pH 6.8 containing 0, 2×10^{-6} and 2×10^{-2} *M* KCN, and the mixtures were kept at 30°. Two test solutions, 0.5 ml. and 1 ml. were pipetted from above solutions at the same time at each time interval in the duration of the incubation. The former soln. was used for assaying the activity after dilution 1700 times and the latter was used for the determination of denaturation by the proteolytic method.

* Complete denaturation was carried out by heating an liquot of the treated solution at 70° for 10 minutes.

** The activity was represented by the unimolecular velocity constant (k) obtained by the diluted enzyme solution.

*** OD_{test} and $OD_{nat.}$ are the optical densities of Folin colour-solutions of the filtrates obtained from the digested solution of the treated samples and non-treated sample, respectively, after addition of TCA at a final concentration of 0.4 *M*. Therefore, the values, OD_{test}-$OD_{nat.}$ show the amount of denatured protein measured by the proteolytic method.

TABLE III

Inactivation and Denaturation of Catalase in the Duration of Treatment with Sodium Fluoride

Conc. of F⁻ (M) / Time of treatment (*min.*)	0			10^{-1}		
	Activity (k)	OD_{test}	$OD_{test}-OD_{nat.}$**	Activity* (k)	OD_{test}	$OD_{test}-OD_{nat.}$**
0	0.030	0.065	0.000	0.029	0.060	−0.005
30	0.030	0.068	0.003	0.030	0.050	−0.015
100	0.029	0.073	0.008	0.031	0.052	−0.013
Complete denaturation	0.000	0.200	0.135	0.000	0.190	0.125

The experiment was carried out similarly to *Expt. 3* (Table II) except that 10^{-1} M sodium fluoride was used instead of potassium cyanide.

* The activity was represented by the unimolecular velocity constant obtained by 1200 times diluted solutions of treated solution.

** The values show the amount of denatured protein.

Experiment 6

Action of Hydroxlamine

The experiment was carried out as in the case of *Experiment 3*, except that hydroxylamine at a final concentration of 2×10^{-3}, 1×10^{-3}, or 4×10^{-4} M was used in the treated solution. As shown in Fig. 4, the same results were obtained as in the case of azide.

Experiment 7

Coupled Action of Denaturing Agent and Speciffic Inhibitor

Since the catalases treated with the above four specific inhibitors were still as resistant to the proteinase as untreated one, it was supposed that the secondary intramolecular structure of catalase was not affected by them. In order to confirm this supposition more clearly, the catalase was treated with one of above inhibitors, sodium azide, in the presence of urea. The time-courses of inactivation and denaturation were shown in Fig. 5 when the catalase was treated with 4 M urea alone, 0.5×10^{-4} M sodium azide alone and both. As shown in Fig. 5, the course of denaturation of the catalase in the urea treatment (Curve A) was not

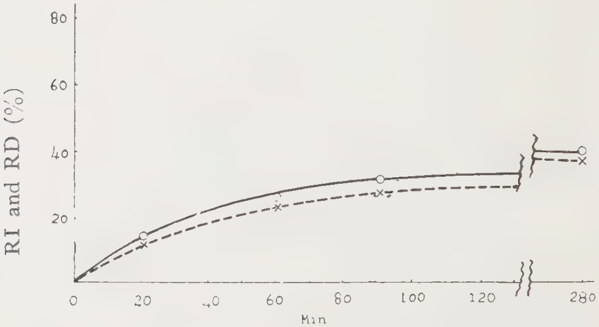

FIG. 2. The action of sodium benzoate on catalase.

About 0.2 per cent catalase solution containing phosphate buffer of pH 6.5 was mixed with the same volume of 1.86 *M* sodium benzoate and the mixture were kept at 20°. The "ratio of inactivation" and "ratio of denaturation" were measured, as in the case of urea-treatment, at each time in the duration of above incubation (see Table I), except that activity of the treated solution was measured after a dilution of approximately 2000 times.

—○— the ratio of inactivation

---×--- the ratio of denaturation

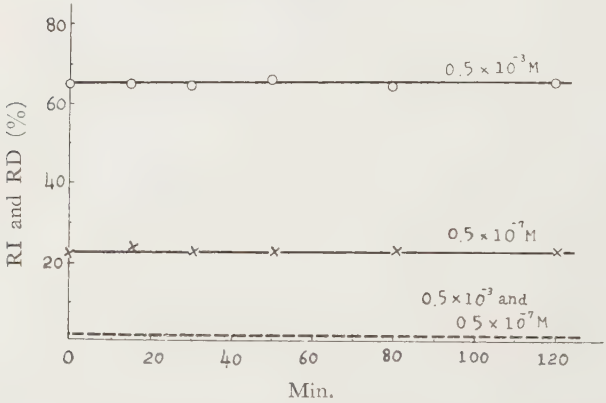

FIG. 3. The inhibition of catalase by sodium azide.

The experimental procedure was similar to *Expt. 3* except that 0.5×10^{-3} *M* and 0.5×10^{-7} *M* sodium azide were used in the treated solution instead of cyanide (see Table II).

Solid line: the ratio of inactivation.

Broken line: the ratio of denaturation.

The assay of catalase activity was carried out after 1,600 times dilution. Temp. 20°.

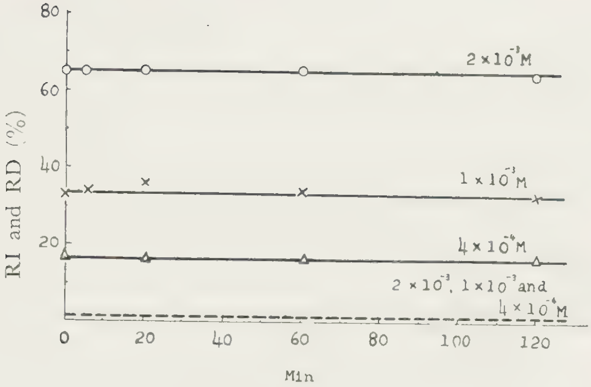

FIG. 4. The inhibition of catalase by hydroxylamine.

The experiment was carried out as *Expt. 3*, except that 2×10^{-3} and 4×10^{-4} *M* hydroxylamine were used instead of cyanide (see Table II).

Solid line: the ratio of inactivation.

Broken line: the ratio of denaturation.

The assay of catalase activity was carried out in a similar manner to that in Fig. 3.

affected by the addition of the azide (Curve C), notwithstanding that the course of inactivation was strongly changed (see Curves a and c). Furthermore, the difference between the " RI " of catalase treated with urea alone (Curve a) and that treated with both urea and azide (Curve c) was almost the same as the value of azide inactivation in the absence of urea (Curve b). From the above results, it was concluded almost certainly that the catalase was not affected by the azide as regards its intramolecular secondary structure.

DISCUSSION

From the construction of the heme protein, many agents which modify the activity of heme enzymes may be classified into following three groups ; 1) Specfic inhibitors which inactivate the heme enzyme by combining with the prosthetic group (heme) alone without any influence to the secondary intramolecular structure of the protein moiety ; 2) Specific inhibitors which combine with heme alone but cause the alteration of the protein moiety by the influence of the above

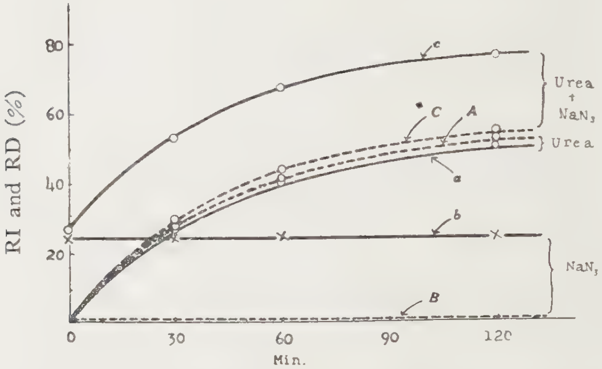

FIG. 5. Effect of sodium azide on the inactivation and denaturation of catalase being treated with urea.

Three different solutions containing 4 *M* urea alone (Curves A and a), 0.5×10^{-4} *M* sodium azide alone (Curves B and b) and both 4 *M* urea and 0.5×10^{-4} *M* sodium azide (Curves C and c) were incubated at 20°. The "RI" and "RD" of the 3 solutions were measured at several stages in the duration of the incubation similarly to the case of urea-treatment (see Table I). In the figures, solid lines (indicated by capital letters) show the courses of inactivation and broken lines (indicated by small letters) show the courses of denaturation.

combination, and thus cause denaturation of the protein moiety; 3) Ordinary denaturing agents which act on the protein moiety alone and cause inactivation of the enzyme by the alteration of the secondary intramolecular structure—denaturation—of the protein.

The first is further divided into two groups, one of which combine quite reversibly with heme and the other which does not combine reversibly. From the results of *Experiments 3–6*, it was concluded that cyanide and fluoride belong to the former group and azide and hydroxylamine belong to the latter. Though it may not be neglected from only these results that the above inhibitions might cause some slight modification of the structure of the protein moiety which could not detected by the proteolytic method, this posibility was almost completely denied by the result of *Experiment 7* in which the time course of denaturation in the urea-treatment was not influenced by the addition of one of the above inhibitors, sodium azide.

The third group may also be divided into two groups, irreversible and reversible denaturing agents, as in the case of the first group. Urea acted as an almost completely irreversible denaturing agent in the condition used in *Experiment 1*. Though the "perturbators" such as benzoate and salicylate have been reported as reversibly modifing agents of the spectrum of heme proteins (*8*, *9*), above agents acted on catalase quite similarly to the urea in the *Experiment 2*. As the recovery of the spectrum of heme protein may not always represent the renaturation of the protein molecule, it was concluded that the agents have behaved as ordinary protein-denaturing agents in the conditions employed. However, the possibility cannot be excluded that the perturbators will act as reversibly denaturing agents when other conditions, especially an other concentration, is employed.

Recently Goldacre and Galston (*10*) have reported the assumption that the specific inhibition of catalase with the substituted phenols was due to direct combination with catalase (heme)-hydogenperoxide complex rather than to nonspecific denaturation of the protein. As the perturbators used in the present investigation have a structure analogy with the above phenol derivatives, it is probable that the same mechanism as above may take place in the denaturation of catalase with the perturbators, i.e., the denaturation may be caused by the influence of the combination of the agents with heme moiety. If the above mechanism is true, the perturbators must belong to the second group mentioned above. However, the above problem could not be solved by the present investigation.

SUMMARY

By assaying the activity and determining the denaturation by the proteolytic method at the same time, the relation between inactivation and denaturation of crystalline beef catalase was investigated in the duration of treatment with urea, perturbators, and four specific inhibitors. The " ratio of inactivation " (RI) and the " ratio of denaturation " (RD) of the catalase almost coincided with one another at any stage in the duration of urea-treatment, but the " RD " was always slightly larger than the " RI " because catalase even native, was slightly digested by the proteinase. So called " perturbators," such as benzoate and salicylate, behaved similarly to urea. Cyanide and fluoride inhibited the enzyme activity without denaturation of the enzyme protein and the inactivated enzyme was completely reactivated by the dilution. Azide and hydroxylamine also inactivated the enzyme without denaturation, but the in-

activated enzyme was not reactivated by the dilution. As the time course of denaturation of catalase in the presence of urea was not changed by the addition of azide, it was concluded that the above specific inhibitors have not affect at all on the secondary intramolecular structure of protein moiety of catalase molecule.

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INTERRELATION BETWEEN THE FUNCTION OF HEME-PROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

VIII. STUDIES ON THE REACTION OF METHEMOGLOBIN WITH FORMALDEHYDE*

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(Received for publication March 28, 1956)

Kikuchi, Suzuki *et al.* (*1*, *2*) in our laboratory reported that the intrinsic oxidase or peroxidase activity which is latent in the native molecule of hemoglobin or catalase was promoted through a slight degree of configurational changes in their protein portions. The combining affinity of azide or fluoride with methemoglobin might also be expected to be affected through similar modifications of its protein portion. In the present experiments, formaldehyde was chosen for the purpose of modifying the protein moiety, because its reaction with amino acids has so far been well explored (*3*).

Concerning the reaction of hemoglobin with formaldehyde, Guthe (*4*) reported that the sigmoid coefficient of oxygen dissociation equilibrium of oxyhemoglobin was decreased by the addition of formaldehyde.

EXPERIMENTAL

Materials

Methemoglobin solution was prepared as follows: Crystalline equine oxyhemoglobin prepared by the method of Suzuki *et al.* (*5*) was dissolved in distilled water and oxidized with sodium nitrite. The excess sodium nitrite was removed by dialysis against distilled water. The concentration of methemoglobin solution was determined by alkali-denatured globin hemochrome method.

Formaldehyde solution adjusted to pH 7.0 with NaOH was diluted at use. The concentration of the formaldehyde solutions was determined iodometrically by the method of Romijin (*6*), every time before use.

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* The content of this paper was read before the 74th Kanto Local Meeting of the Biochemical Society of Japan in January, 1955, at Tokyo.

Sodium azide and sodium fluoride of Merck's reagents were dissolved in desired concentrations in buffer solutions of various pH.

Methods

All reactions were carried out in a small beaker of about 20 ml. content at room temperature. A photoelectric spectrophotometer Model EPB-V of Hitachi Co. Ltd. was used for this experiments. pH of the reaction mixtures containing carbonate, phosphate, and acetate buffers of $M/5$, $M/15$, $M/5$, respectively, were adjusted by using a glass electrode.

RESULTS

Reaction of N_3-Methemoglobin with Formaldehyde—The absorption figure of methemoglobin at pH 6.5 changed to that of N_3-methemoglobin by the addition of sodium azide in a concentration of 10^{-3} M, as shown in Fig. 1, Curve I. This absorption of N_3-methemoglobin changed toward a more indistinct absorption figures (Fig. 1, Curve V in the final state) by the addition of formaldehyde in increasing concentrations, demonstrating clearly two isosbestic points along these absorption changes as shown in Fig. 1 (II–IV). It may thus be assumed that Curve V in Fig. 1, is the final figure of this two-componental reaction system consisting of N_3-methemoglobin and formaldehyde-methemoglobin, was the same as that which could be obtained by the addition of 1.0 M formaldehyde to methemoglobin without the presence of azide. It should be noted that this figure is also the same as that of acid methemoglobin at pH 6.5, irrespective of the presence of formaldehyde; the absorption figure of formaldehyde-methemoglobin was thus indicated to be identical with that of acid methemoglobin.

The absorption figures shown in Fig. 1, Curves II–V were reversed rapidly to that of N_3-methemoglobin by the addition of excess sodium azide.

Potassium cyanide was also effective in a similar manner. This may be due to the decrease of formaldehyde concentration as a result of the removal of formaldehyde from the reaction system, reacting with cyanide to produce cyanohydrin.

From these qualitative observations, it seems that the reaction of N_3-methemoglobin with formaldehyde is a competitive bonding of azide with formaldehyde toward heme–Fe^{III} of methemoglobin.

Quantitative Observations on the Reaction of N_3-Methemoglobin with Formaldehyde—Based on the qualitative observations, the effect of formal-

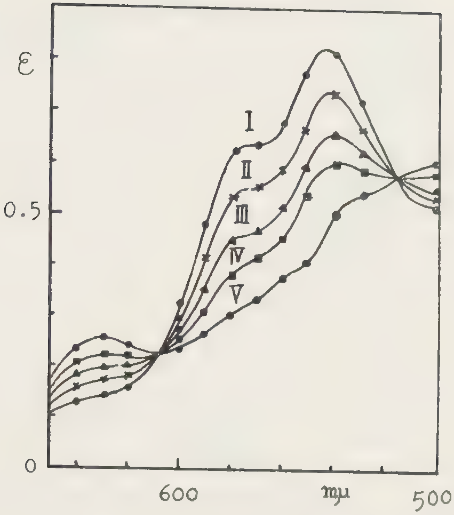

FIG. 1. Absorption changes of N_3-methemoglobin by the addition of formaldehyde in varied concentrations.

Methemoglobin: 6.75×10^{-5} *M*
Sodium azide: 10^{-3} *M*
Formaldehyde: I. 0 *M*. II. 5.5×10^{-1} *M*. III. 8.0×10^{-1} *M*
IV. 1.0 *M*. V. Methemoglobin + 1*M* HCHO
pH: 6.5

dehyde on the combining affinity of azide for methemoglobin was quantitatively followed.

As mentioned above, the absorption figure of N_3-methemoglobin changed, showing two isosbestic points, gradually toward the final figure (Fig. 1, V) by the increasing addition of formaldehyde. Then, taking Curve V as the final limit, the percentages of the decrease of N_3-methemoglobin concentration were calculated from the nomogram obtainable from Fig. 1. The percentages of the decrease were then plotted against the log concentrations of formaldehyde as shown in Fig. 2. A sigmoid curve of a second order reaction with respect to formaldehyde concentration was recorded. Thus, the following equilibrium equation was postulated presumably for the reaction of N_3-methemoglobin with formaldehyde.

$$N_3\text{-metHb} + 2\ \text{HCHO} \rightleftharpoons \text{metHb(HCHO)}_2 + N_3^-$$

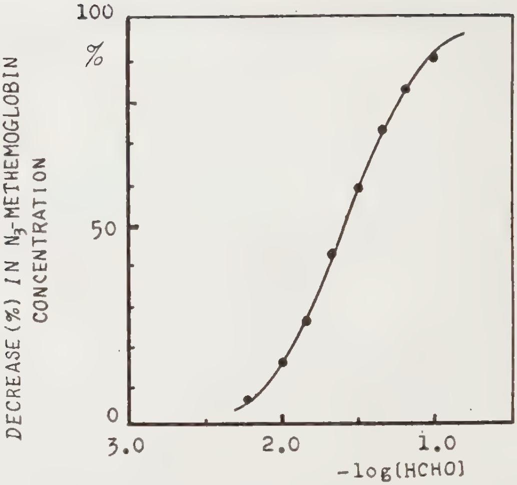

Fig. 2. Plots of percentages of decrease in N_3-methemoglobin concentration *vs* log concentration of formaldehyde.

Reversibility of the Reaction of N_3-Methemoglobin with Formaldehyde— To examine the reverse reaction, the following experiments were carried out.

Formaldehyde in varied concentrations was preliminary added to methemoglobin solution and then sodium azide was added in varied concentrations at pH 6.0. The percentages of N_3-methemoglobin formation were calculated from the absorption change of methemoglobin into N_3-methemoglobin. These values were plotted against the log concentrations of free sodium azide as shown in Fig. 3. Sigmoid curves of first order reaction with respect to sodium azide concentrations were obtained.

From the result, it was ascertained that the reaction of methemoglobin with sodium azide in the presence of formaldehyde proceeded in accordance with the equation postulated above.

When the concentrations of free sodium azide at onehalf formation of N_3-methemoglobin was taken as an apparent dissociation constant (K') in the reaction of methemoglobin with azide in the presence of formaldehyde of various concentrations, it was observed, as shown in Fig. 3, that the pK' decreased with the increase of formaldehyde concentrations. The pK' values were plotted against the log concentrations

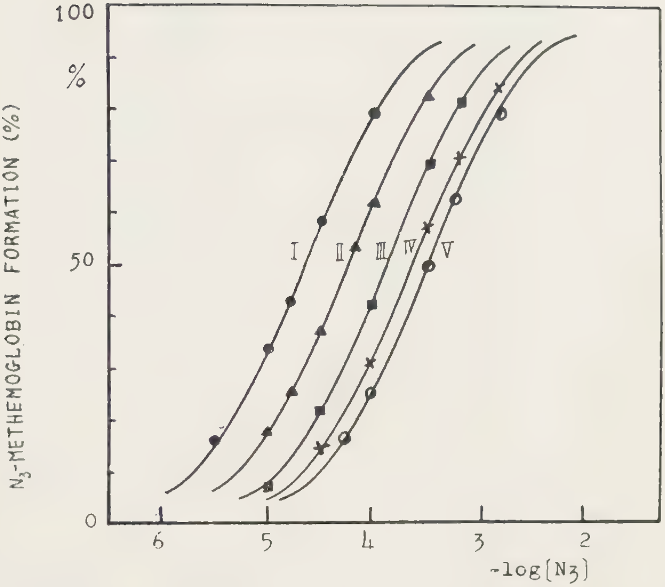

FIG. 3. Plots of percentages of N_3-methemoglobin formation *vs* log concentration of azide in the presence of various concentration of formaldehyde.

Methemoglobin: 7.25×10^{-5} *M*

Formaldehyde: I. 0.2 *M*. II. 0.4 *M*. III. 0.6 *M*. IV. 0.8 *M* V. 1.0 *M*.

pH. 6.0

of formaldehyde and gave a straight line as shown in Fig. 4. The slope of this line was calculated as 2 reasonably, giving further support to the assumption that the reaction of N_3-methemoglobin with formaldehyde proceeds in accordance with the above equation.

Effect of pH on the Reaction of N_3-Methemoglobin with Formaldehyde—The foregoing reactions were carried out at varied hydrogen ion concentrations. Plotting of the percentages of N_3-methemoglobin formation against log concentrations of sodium azide in the presence of various formaldehyde concentrations at each pH appeared as sigmoid curves of first order.

pK′ at each pH was plotted against log concentrations of formaldehyde in Fig. 5. Within a definite range of formaldehyde concen-

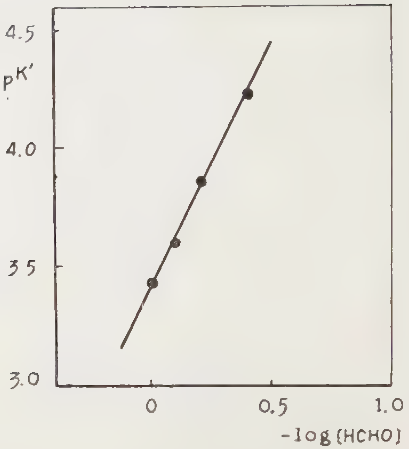

FIG. 4. Plots of pK′ against log concentration of formaldehyde.
pH: 6.0

trations, each plot was recorded as a straight line. These lines were parallel with each other, indicating a second order reaction with respect to the formaldehyde concentrations. Accordingly, it was noted that in a range of pH from 5.0 to 7.0, the reaction of N_3-methemoglobin with formaldehyde proceeded according to the above equation at a definite formaldehyde concentrations.

The pK′ values of reaction system at infinite concentration of formaldehyde (1.0 *M*), which obeys the above equation, were plotted against the pH as shown in Fig. 6. In a pH range of 5.0–7.0 the pK′ values fall with the increase of pH.

Jung *et al.* (7) reported that the pK values of the reaction of sodium azide with methemoglobin decreased lineally with the increase of pH in a pH range of 6.0–8.0, as shown by the broken line in Fig. 6. Comparing these two curves, it was assumed that not only the reaction of methemoglobin with sodium azide, but also the reaction of methemoglobin with formaldehyde was affective by the hydrogen ion concentration.

Reactions of F-Methemoglobin with Formaldehyde—The absorption figure of methemoglobin at pH 6.0 in the presence of 0.2 *M* formaldehyde was converted into that of F-methemoglobin by the addition of sodium fluoride in varied concentrations, as shown in Fig. 7, Curves I–VII.

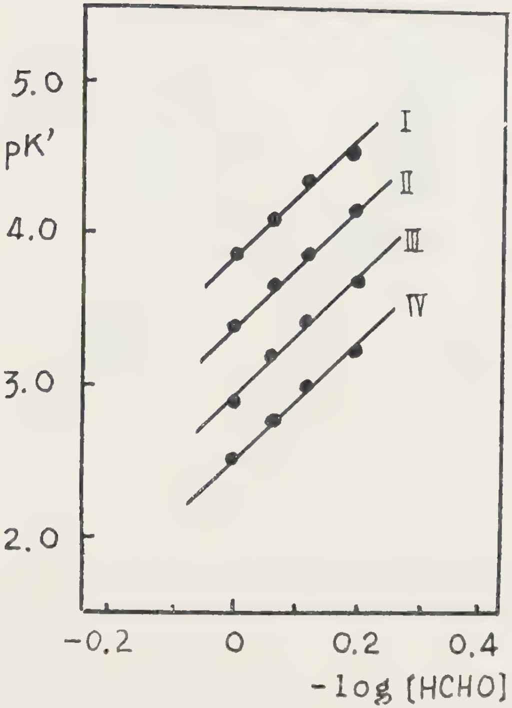

FIG. 5. Plots of pK′ against log concentration of formaldehyde at various pH.
pH: I. 5.0, II. 6.0, III. 6.5, IV. 7.0

The absorption figure represented by Curve VII is that of F-methemoglobin. Two isosbestic points were demonstrated in this figure indicating a reaction system of two components. Then, the percentages of F-methemoglobin formation was represented as a function of fluoride concentration, giving a sigmoid curve shown in Fig. 8. The sigmoid coefficient was found to be one indicating a reaction of first order.

The pK′ in the reaction of methemoglobin with fluoride in the presence of formaldehyde at pH 6.5 were plotted against log concentration of formaldehyde, indicating a straight line in a definite formaldehyde concentration range as shown in Fig. 9. The slope of this line was calculated as 2.

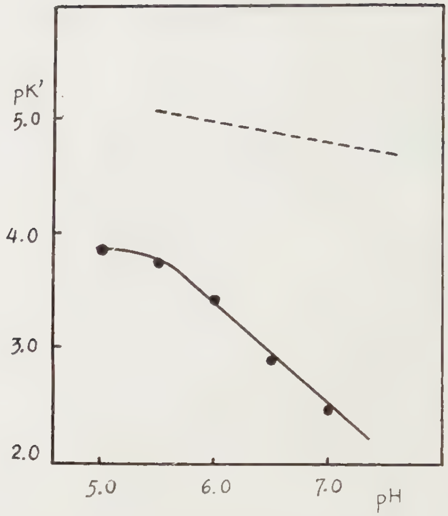

FIG. 6. Plots of pK' against pH.
Broken line indicates a relationship between pK of N_3-methemoglobin and pH (reported by Jung).

From these results, it was assumed that the reaction process of methemoglobin with fluoride in the presence of formaldehyde was the same as that of methemoglobin with azide.

Above reactions were carried out at different hydrogen ion concentrations. Plotting of pK' values against log concentration of formaldehyde in varied pH is shown in Fig. 10. Within a definite formaldehyde concentration range, each plot was recorded as a straight line. These lines were parallel with each other indicating a second order reaction with respect to the formaldehyde concentrations.

The pK' values at the 1.0 *M* formaldehyde concentration, which were obtained by exptrapolation of the straight line in each pH, were plotted against pH as shown in Fig. 11.

Reaction of Methemoglobin with Formaldehyde in Alkaline Medium—The absorption figure of methemoglobin at pH 9.2 is shown in Fig. 12. This absorption figure changed gradually toward a more indistinct absorption figure with increasing formaldehyde concentration as shown in Fig. 12 Curve (I–VI). Above 1.0 *M*, no further absorption change was observable. Curve VI could thus be regarded as a final

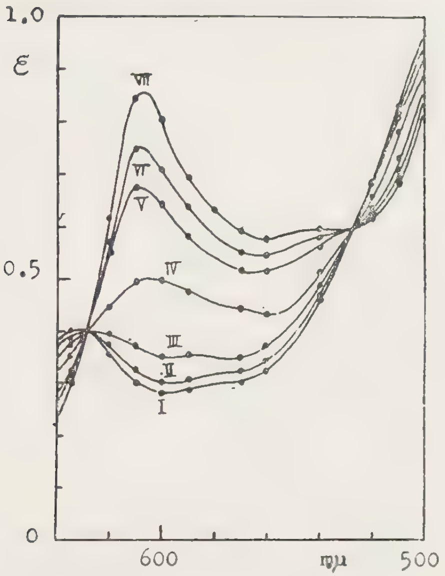

Fig. 7. Absorption changes of methemoglobin in the presence of formaldehyde by the addition of sodium azide.
Methemoglobin: 9.12×10^{-5} *M*
Formaldehyde: 0.2 *M*
Sodium fluoride: I. 0 *M*., II. 3×10^{-3} *M*., III. 7×10^{-3} *M*., IV. 2.5×10^{-2} *M*., V. 1.5×10^{-1} *M*., VI. 2.5×10^{-1} *M*., VII. Methemoglobin$+5 \times 10^{-1}$ *M* NaF
pH: 6.0

limit of the absorption change. Based on this absorption change, the relationship between the formation of methemoglobin-formaldehyde compound and log concentration of formaldehyde is shown in Fig. 13, indicating a sigmoid curve of the first order reaction. Methemoglobin in the presence of 1.0 *M* formaldehyde at pH 9.2 showed no absorption change by the addition of 10^{-2} *M* sodium azide. When the pH of this reaction mixture was brought to 6.0, however, there arose an absorption figure of N_3-methemoglobin as shown by the broken line in Fig. 12. When, however, the reaction mixture containing methemoglobin, 1.0 *M* formaldehyde and 10^{-2} *M* azide at pH 9.2 was allowed to stand during several hours at room temperature, no N_3-methemoglobin was formed, even after adjusting the pH to 6.5.

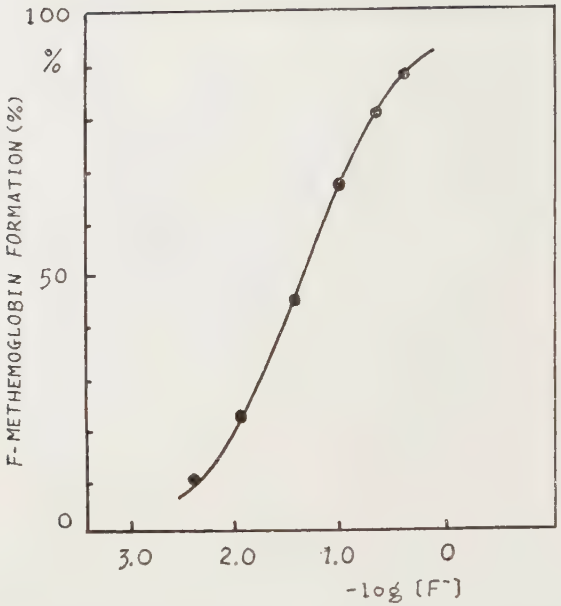

FIG. 8. Plots of the percentage of F-methemoglobin formation against log concentration of sodium fluoride.

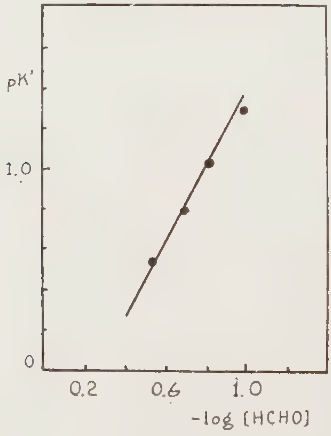

FIG. 9. Plots of pK′ against log concentration of formaldehyde.

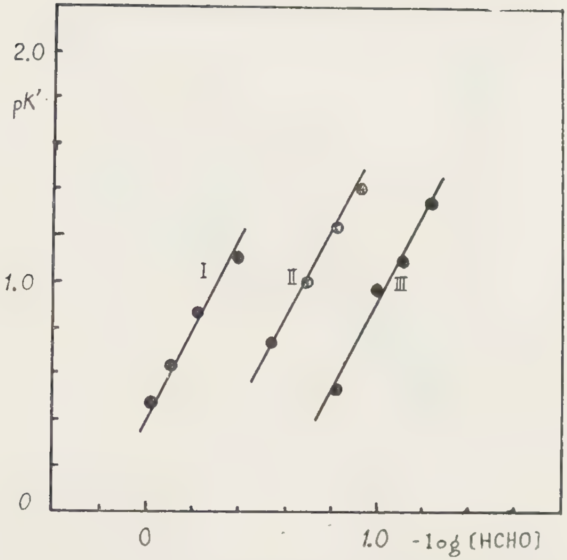

FIG. 10. Plots of pK′ against log concentration of formaldehyde at various pH.

pH: I. 6.0, II. 6.5, III. 7.0

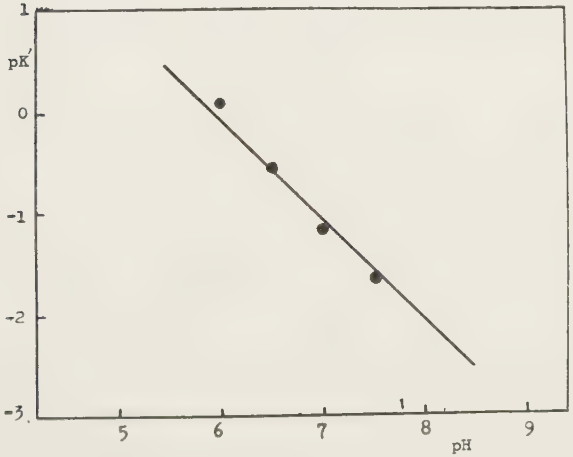

FIG. 11. Plots of pK′ against pH.

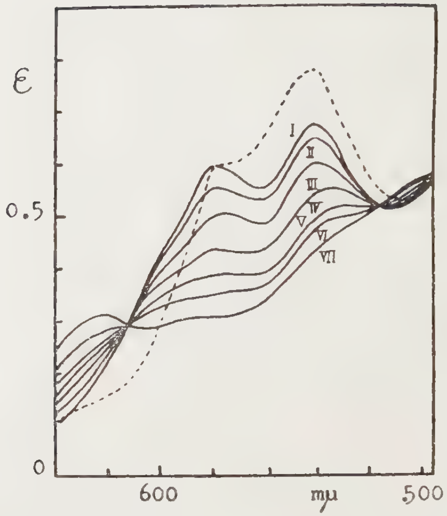

FIG. 12. Absorption changes of alkali methemoglobin by the addition of formaldehyde in varied concentrations.

Methemoglbin: 6.75×10^{-5} *M*

Formaldehyde: I. 0 *M*. II. 10^{-2} *M*. III. 2×10^{-3} *M*. IV. 5×10^{-2} *M*. V. 10^{-3} *M*. VI. 2×10^{-1} *M*. VII. 2×10^{-3} *M*. 3×10^{-1} *M*

Broken line indicates the absorption figure of N_3-methemoglobin

DISCUSSION

From the experimental results of the reactions of N_3-methemoglobin or F-methemoglobin with formaldehyde described above, it seems likely that two formaldehyde molecules combine reversibly with heme Fe^{III} of methemoglobin, competing with one molecule of azide of fluoride. From the fact, however, that the absorption of acid methemoglobin was not altered by the addition of formaldehyde, it is presumed that formaldehyde does not combine appreciably with the heme Fe^{III} of methemoglobin. Thus it may be assumed that the effect of formaldehyde would rather be concerned with the amino, imino, or sulfhydryl group of protein moiety. The exact nature of the groups of protein portion involved still remains to be found, but it is assumed at least that the two protein groups involved in the reversible binding of formaldehyde interact directly with heme Fe. Which of these groups in the protein portion

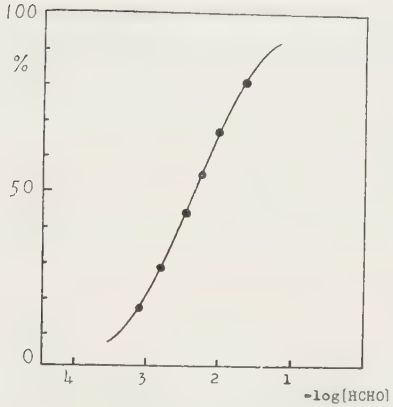

FIG. 13. Plots of percentage of decrease in alkali methemoglobin concentration against log concentration of Formaldehyde.

would take part is not clear, but from the range of pH in which the formaldehyde is effective, it may be suggested that these groups may be imidazoles, which have the pK values of 6.68 and 5.75, respectively, as reported by Wyman *et al.* (*8*).

The absorption figure obtained by the addition of formaldehyde to methemoglobin at pH 9.2 resembles that of acid methemoglobin, but this absorption figure is not completely in agreement with that of acid methemoglobin. On the chemical structure of the heme portion in alkaline medium in the presence of formaldehyde, further investigations are needed.

SUMMARY

1. The combining affinity of methemoglobin for azide or fluoride decreased reversibly by the addition of formaldehyde.

2. The reaction of N_3-methemoglobin with formaldehyde was found to proceed according to the following equation.

$$\text{N}_3\text{-metHb} + 2\ \text{HCHO} \rightleftharpoons \text{metHb(HCHO)}_2 + \text{N}_3^-$$
$$\text{F-metHb} + 2\ \text{HCHO} \rightleftharpoons \text{metHb(HCHO)}_2 + \text{F}^-$$

3. The pK′ values of methemoglobin-azide equilibrium in the presence of formaldehyde fall gradually as the pH increased, in the range of 5.0 to 7.0.

4. The absorption figure of acid methemoglobin was not altered by the addition of formaldehyde, but the absorption figure of alkali methemoglobin at pH 9.2 changed by the addition of formaldehyde.

5. It was assumed that two imdazole groups may combine with formaldehydes so as to cause a decrease in the binding affinity of methemoglobin with azide or fluoride.

The author wishes to thank Profs. K. Kaziro and G. Kikuchi for their continous guidance and encouragement during the course of this work.

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THE QUANTITATIVE DETERMINATION OF AMINO ACIDS BY PAPER CHROMATOGRAPHY

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(Received for publication, April 6, 1956)

Since the attempts by Polson (*1*), Naftalin (*2*) and Fisher, Parsons and Morrison (*3*) to use spot colour intensity and spot area for the quantitative determination of amino acids on paper chromatograms, a variety of methods for quantitative assay has been reported, and it is now generally recognized that such methods give only semi-quantitative data.

We describe in this paper a method dependent on spot area and colour intensity which has a high precision, and may be of use in circumstances in which absorptiometers and densitometers are not available.

EXPERIMENTAL

Ascending cromatography was carried out in 40×40 cm. sheets (No. 50 paper: Toyo Filter Paper Co.), using *n*-butanol-acetic acid-water (4:1:2). The amino acids were applied as 2.5/μl. spots, 2.5 cm from the paper edge. After development the paper was allowed to dry at air temperature, and a 0.2 per cent ninhydrin solution was sprayed evenly over it, taking particular care, a) that series of spots of the same amino acid were similarly sprayed, and b) that no part of the paper was grossly wetted. The paper was left at room temperature for 1/2–1 hour after spraying and then inserted for exactly 2 minutes into a heating apparatus at 136°. As shown in Fig. 1. It consists of a rectangular chamber with heating elements under its base, and there is a horizontal slit for insertion of the paper so that it lies on the base. There should be a door or doors to close the slit.

RESULTS

A series of spots of serial dilutions of an amino acid solution were placed on paper, and the colour developed as described above, without chromatography. This paper formed the standard colour paper for that amino acid. This paper was used to mark the chromatograms in the manner shown in Fig. 2, where the innermost boundary indicates

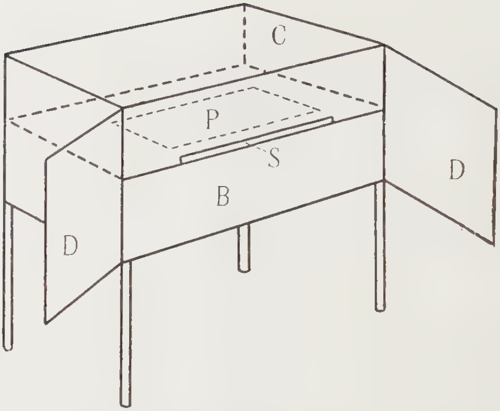

Fig. 1. Diagram of chromatogram heating apparatus: C, heating chamber, P, paper chromatogram on base of chamber, B, base containing heating elements, S, slit for introduction of paper, D, doors to cover slit.

the line at which the colour intensity falls below the highest standard colour represented, the next boundary where it falls below the next highest intensity represented, and so on.

The outlining of intensity regions is done in a dark room by transmitted light and the areas in each region are measured by square-counting on graph paper. Each area is multiplied by the dilution of the standard amino acid solution used on the standard paper and the sum of these quantities is taken as the " size " of the spot.

The chromatogram shown in Fig. 2 was prepared by putting on the paper successive dilutions in the ratio of 9 :10 of a synthetic mixture of amino acids. The " size " of spots obtained from chromatograms of this kind is shown in Table I. for the three amino acids leucine, phenylalanine and valine. Although there are variations in spot " size " from instance to instance involving the same concentration of the same amino acid, inspection shows that particularly aberrant values at one concentration are in general accompanied by similar aberrations at other concentrations on the same paper, suggesting that the variations are largely due to differences in paper or treatment in different runs.

The mean spot size was calculated for each set of five chromatograms, Table II shows that, when this is divided by the concentration of amino acid, it yields an almost constant quantity for each amino-

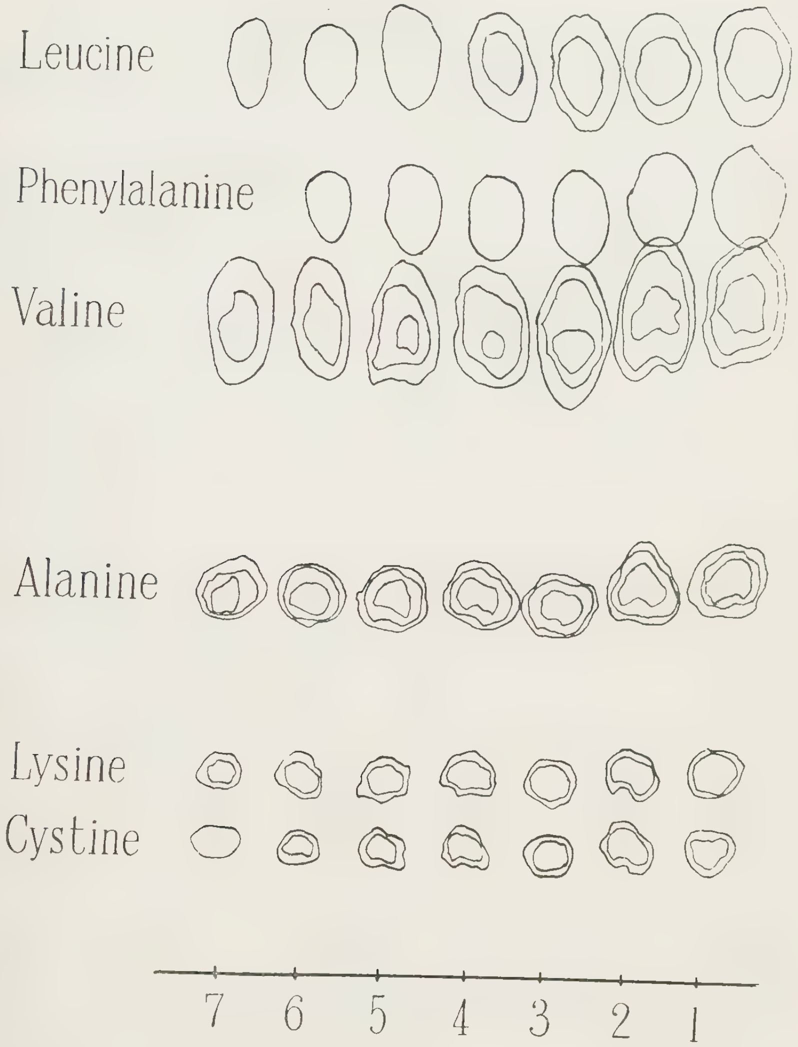

Fig. 2. Chromatogram of spots made by successive dilutions of stock mixture by a factor of 0.9G Dilutions 1, 500 μg./ml. of each amino-acid; dilution 7. $500 \times 0.9^6 = 269$ μg./ml.

acid. It is, therefore, reasonable to suppose that a quantitative method

TABLE I

The Relation between " Size " of Chromatogram Spots and the Concentration of Amino Acid

Amino-acid	Paper	" Size " of spots (arbitrary units) at successive dilutions of amino-acid solutions.				
		1	(0.9)	$(0.9)^2$	$(0.9)^3$	$(0.9)^4$
Leucine	1	783	684	581	551	432
	2	807	742	716	578	511
	3	840	754	659	574	469
	4	689	721	598	587	503
	5	752	736	595	511	431
Phenylalanine	1	339	357	324	258	210
	2	441	355	317	305	246
	3	586	522	508	427	360
	4	436	477	435	400	353
	5	461	392	391	363	280
Valine	1	540	441	372	358	289
	2	443	376	351	316	269
	3	610	595	569	560	359
	4	789	677	640	532	482
	5	663	678	574	446	392

Amino acids were applied to chromatograms as 2.5 μl.-spots, and stock solutions contained 2000 μg./ml.

TABLE II

Ratio of Spot " Size " to Dilution of Amino Acid Solution
(data of Table I)

Amino-acid	Mean ratio of spot size to dilution of amino-acid solution for successive dilutions.				
	1	(0.9)	$(0.9)^2$	$(0.9)^3$	$(0.9)^4$
Leucine	774	808	778	768	715
Phenylalanine	453	467	488	481	442
Valine	609	615	619	607	546

can be developed on the assumption of proportionality between spot " size " and spot content of amino acid. Smaller numbers of measurements of spots of alanine, glutamic acid, aspartic acid, cystine and lysine fit this relation.

Quantitative Method—This is best illustrated by an example. Consider a single amino-acid. Replicate series of the unknown solution and successive 1 : 2 dilutions of it down to 1 : 8 are run side by side with replicates of standard solution and of 1 : 2 dilution of the standard. After colour development it may be seen by inspection which of the unknown dilutions is bracketed by the standards. If both standards are too strong, this procedure must be repeated with further standard dilutions.

A second chromatogram is then run with replicates of the bracketed unknown dilution in comparison with replicate series of 9 : 10 dilutions of the higher of the bracketing standards. (Five such dilutions cover the range between 1 and ½ times the concentration of the higher standard dilutions.) Inspection will show the unknown spots to fall between two of the standard dilutions. Sets of four dilutions in the ratio 9 : 10 are now prepared for unknown and standard, starting with the unknown dilution used in the previous chromatogram and with the standard dilution which gave a just larger spot on that chromatogram, and these are run in duplicate on a third chromatogram. The product of " size " and dilution is computed for every spot, and the mean found for standard and unknown series. In taking means, any grossly aberrant values are excluded. The amino acid content of the unknown solution is then found by simple proportion.

As an indication of the reliability of this method mixtures were analysed which had been prepared by another worker, so that their composition was unknown to the analyst. The mixtures contained alanine, valine, phenylalanine, leucine, cystine, tyrosine and proline, which can be separated by the butanol-acetic acid mixture. Proline spots were developed with 0.2 per cent isatin in *n*-butanol containing 4 per cent acetic acid. The results of these analyses are presented in Table III. They show quite clearly that the method is applicable to all the amino-acids used and that it yields estimates of a high order of accuracy.

Table IV gives the mean percentage errors and the standard deviations of the percentage errors for the five amino acids shown in Table III for which there are sufficient observations. It will be seen that, except in the case of lysine, the mean percentage errors are very small indeed, and do not differ significantly from zero. The standard devi-

TABLE III

Recovery of Amino Acids from Synthetic Unknown Mixtures

	Concentrations of amino acids (μg./ml.)						
Theoretical:	545.4	600.0	625.0	714.3	750.0	800.0	857.1
Found:							
Lysine	533.0	......	613.1	699.9	......	793.8	843.2
Alanine	547.1	......	616.1	712.5	......	796.9	857.8
Valine	546.7	......	622.2	721.6	......	805.8	849.1
Phenylalanine	548.9	......	622.0	721.7	......	787.8	866.6
Tyrosine	......	605.3	......	......	......	821.7	......
Cystine	......	602.6	......	......	......	794.5	......
Proline	......	......	......	......	773.9	......	857.8

TABLE IV

Percentage Errors of Recovery of Amino Acids from Synthetic Mixtures with Mean Errors and Standard Deviations

Amino acid	Percentage errors of recovery					Mean and standard deviations of percent errors	
Lysine	−2.28	−1.90	−2.01	−0.77	−1.62	−1.72	±0.61
Alanine	+0.31	−1.41	−0.24	−0.40	0.00	−0.35	±0.63
Valine	+0.24	−0.44	+1.02	+0.72	−0.93	+0.03	±0.82
Phenylalanine	+1.63	+2.47	−1.02	−3.07	−1.01	−0.21	±2.24
Leucine	+0.64	−0.48	+1.03	−1.52	+1.11	+0.16	±1.13

ations, which measure the reproducibility of the estimations, are much smaller than those found by other workers using related methods, and average just over 1 per cent.

Estimations of glutamic acid on chromatograms run in phenol water (85 :15) showed an error of recovery of about 10 per cent. Aspartic acid could be estimated fairly successfully on butanol-acetic acid chromatograms, since it gave a purple colour with ninhydrin and the adjacent spots gave russet ones. The error of estimation was about 5 per cent.

DISCUSSION

The method described here compares very favourably with previously reported attempts to estimate amino-acid concentrations on paper. Fisher *et al.* (*3*) suggested that a high precision could be obtained (2–4 per cent error) with sufficient replications, of an area measurement procedure. Bull, Hahn and Baptist (*4*), using a photometric scanning method, report a coefficient of variation of ±7.47 per cent. Redfield and Barron (*5*), using two variants of a similar method, report coefficients of variation of ±7.47 per cent and ±4.36 per cent. Hiller, Zinnert and Frese (*6*) again using photometric scanning, report results which give a coefficient of variation of ±3.04 per cent. Fowden (*7*) has used an elution method, and it is possible to compute for three of his series coefficients of variation of ±2.38, 3.82 and 4.56 per cent. The present results compare favourably with these. The pooled coefficient of variation for the series of observations reported in Table IV is ±1.24 per cent. It seems probable that the greater accuracy of our procedure depends on the use of successive approximations.

SUMMARY

A method has been described for accurate estimation of amino acids on paper chromatograms.

It consists of, a) visual comparisons of series of spots obtained from dilutions of unknown and standard solutions to give an estimate of the unknown in terms of standard to within 5 per cent, and b) a more exact comparison of closely similar dilutions of standard and unknown in terms of a special technique of measurement of spot size.

The coefficient of variation of individual estimations by this method is ±1.24 per cent.

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ION-EXCHANGE CHROMATOGRAPHY OF AMINO ACIDS AND PEPTIDES

I. A NEW DETERMINATION METHOD OF BASIC AMINO ACIDS ON A CARBOXYLIC ACID RESIN*

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As a method of amino acid analysis, an ingenious technique of Moore and Stein (*1*) using ion-exchange chromatography is probably the most efficient yet developed. According to their method, most of naturally occurring amino acids except basic ones are separated by elution analysis on a 0.9×100 cm. column of a sulphonated polystyrene resin (Dowex 50–X8), and the amino acid content in each of the effluent fractions is estimated by the photometric ninhydrin method (*2*). Basic amino acids, on the other hand, can scarcely be determined quantitatively by the method, and are therefore analyzed in a separate experiment with a shorter column (0.9×15 cm.) of the same resin. Although such a method is excellent in its high resolving power and accuracy, it is rather laborious in the points of each determination of hundreds effluent fractions and other operations. The present author made efforts to simplify the method leaving the point of excellence as it is, and this trial has succeeded in the analysis of basic amino acids by use of a less acidic exchanger, Amberlite IRC-50 (XE-64) instead. In the method of Moore and Stein above mentioned for separation of basic amino acids, three kinds of buffers of progressively increasing elution power must be stepwise used as an eluent and the elution continued until 200 ml. of effluent flows, while in the present method only about 50 ml. of a single eluent is required.

Moore and Stein recently reported a modified method (*3*) which made it possible to analyze quantitatively the basic amino acids simultaneously with the others on a single column (150×0.9 cm.) of Dowex

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* The present paper was partly read by the author before the Symposium on Biological Chemistry held on Oct. 25, 1953 in the University of Tokyo.

50–X4 using an eluent of gradually increasing pH and ionic strength. Nevertheless, when analysis for the basic amino acids is the main object, the present method will be found far more convenient.

PROCEDURE

Eluent—A mixture of 2 volumes of 0.2 *M* sodium citrate buffer (pH 5.95–6.00, 0.575 *N* in Na^+) and 1 volume of 0.2 *M* sodium phosphate buffer (pH 5.95–6.00, 0.236 *N* in Na^+) was used. The citrate buffer was prepared by dissolving 42.0 g. of $C_3H_4(OH)(COOH)_3 \cdot H_2O$ and 23.0 g. of NaOH, the phosphate buffer by dissolving 25.6 g. of $NaH_2PO_4 \cdot 2H_2O$ and 12.9 g. of $Na_2HPO_4 \cdot 12H_2O$, in water and by diluting each solution to a volume of 1 liter. The pH value of each buffer was measured with a Beckman model G pH-meter to assure that it was within pH 5.95–6.00. If not, it was adjusted by using *N* NaOH or *N* HCl. The buffers were stored in the cold after adding a small quantity of thymol as a preservative. They were mixed together in the ratio above indicated. The mixture, which in the following will be called the eluent buffer, may also be stored in the cold.

Preparation of Resin—The resin used in this experiment was of carboxylic acid type, Amberlite XE-64 which is a pulverized form of IRC-50. In a similar manner with the description by Hirs *et al.* (*4*), the resin was washed with water, acetone, alkali (NaOH) and acid (3 *N* HCl). The washed resin was buffered at pH 5.95–6.00 in the following process. The resin of the acid form was stirred with about 3 volumes of the eluent buffer. The pH of the suspension fell immediately and 5 *N* NaOH was gradually added to keep it at about 6. When the suspension remained at pH 6.0 after 15 minutes of stirring, it was left overnight. If the pH was constantly kept at 5.95-6.00, the resin was transferred to a glass filter and washed with the same buffer. The pH of the final filtrate should be equal to that of the influent. After being suspended in the buffer and sealed tightly, the resin thus prepared can be stored in a dark and cold place. The resin in storing however showed a little tendency to have a higher blank value in the analysis.

Preparation of Column—The chromatographic tube used was a glass one with a coarse sintered glass plate near the bottom (0.6 cm. in inner diameter and 40 cm. in length above the plate). The jacket (35 cm. in length and 3 cm. in outer diameter), similar to that of a condenser, was attached to the tube. The buffered resin was suspended in about 2 volumes of the eluent buffer and any bubble was exhausted from the suspension by stirring gently. The suspension was then poured into the tube evenly through a tip-bent funnel (see Moore and Stein (*1*)). In this way the column of resin with 30 cm. in height was made.

Operation of Column—During the following operation water of 30°±0.5° was circulated through the jacket. A separatory funnel containing the eluent buffer was jointed upon the tube. After about 10 ml. of the buffer had been run through (pH of the filtrate must be, of course, equal to that of the eluent), the column was ready for use.

A convenient load for analytical work is about 1 μmole in each basic amino acid. Since the capacity of the resin is very high, the load can be increased several fold if necessary. When an acid hydrolysate of protein should be analyzed, it must be evaporated to dryness *in vacuo* to remove most of hydrochloric acid and the residue was then dissolved in a suitable volume of the eluent buffer. After the supernatant buffer was withdrawn from the column, 0.5–1 ml. of sample solution containing appropriate amounts of the amino acids in the buffer was added. After this solution and three washings with the buffer of 0.3 ml. each were drained into the column, the tube was filled with the buffer, the funnel was jointed again and elution was started at a flow rate of about 2 ml. per hour. The flow rate was conveniently controlled by applying suitably reduced pressure to the top of the funnel. All through the operations, the surface of the resin bed must not be left dry for fear that bubbles of air should enter. The column was mounted on an automatic fraction collector* and, collecting each fraction in 0.8 ml., the elution was continued till the 65th fraction (52 ml. in total). Each of the effluent fractions was brought to pH 5 with acid (0.2 ml. of 6/16 N HCl) and analyzed for amino acids by the photometric ninhydrin method in which the modified condition of Cocking and Yemm (*6*) was used excepting that a heating period of 15 minutes was doubled. The optical densities at 570 mμ of the resulting colours were read on a Beckman DU spectrophotometer.

The resin column, so far as simple mixtures of amino acids are applied as a sample, may be used successively in the next analysis after some washing with the buffer. When the column is coloured or the blank value of the effluent increases to an inadmissible degree, the resin must be withdrawn, washed with 3 N HCl and buffered again.

RESULTS AND DISCUSSION

The synthetic mixture of lysine, histidine, arginine, ammonia (as sulphate), valine and tryptophan (1 μmole each) was analyzed by this method, whose result is given as an elution curve in Fig. 1. It is shown that the separation of the amino acids including ammonia is complete. Each amino acid and ammonia were determined with recoveries of 100 ±3 per cent. All neutral and acidic amino acids were found to emerge near the position of the valine peak, while ornithine overlapped on the lysine peak.

This result was obtained at 30°, while at the lower temperature only the position of histidine was delayed as shown in Table I and the resolution of arginine and histidine was liable to be incomplete. Table

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* The fraction collector used in the present study was of a magnetic burette type controlled photoelectrically which was designed by Mr. Takaaki Tamura in our laboratory and manufactured by Tokyo Kagaku-Seiki Co. The accuracy is within ±3 per cent in the case of 1 ml. collection (*5*).

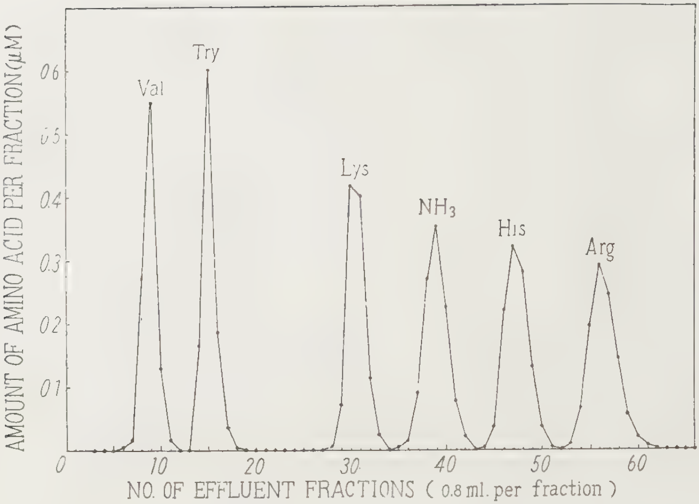

Fig. 1. Chromatographic separation of a mixture containing lysine, histidine, arginine, ammonia, valine and tryptophan on a column of Amberlite IRC-50 (XE-64).

All neutral and acidic amino acids emerge near the position of the valine peak. Column dimensions: 0.6 × 30 cm. Eluent: a mixture of 2 volumes of citrate buffer and 1 volume of phosphate buffer (0.2 *M*, pH 6.0 in each). Load: 1 μ mole per amino acid. Flow rate: 2 ml. per hour. Temperature: 30°.

I indicates the effect of temperature on the position of emergence of each peak, and also, for reference, the similar effects which were observed using 0.2 *M* citrate buffers (pH 6.0 and 6.5) as the eluent instead. (The latter results were obtained with the resin columns previously buffered by each eluent concerned.)

It was indicated, on the other hand, when the proportion of the phosphate buffer in the eluent was increased, the rate of travel of each amino acid showed a tendency to be decreased, which was particularly striking in arginine. This phenomenon may partly be resulted from the decrease of concentration of Na^+ (Na^+ concentration in each of the buffers has already been mentioned), but the specific behaviour of arginine may hardly be explained without considering the change of composition of the anions in the eluent. When the operation is obliged to be carried out at the lower temperature, the content of the phosphate

TABLE I

Effluent Volumes to Peak of Each Amino Acid at Various Temperatures

The amino acids are eluted from a 0.6×30 cm. column (volume of resin, 8.5 ml.) by the standard eluent or citrate buffers.

Eluent	Temp., (°C)	Effluent volume to peak (*ml.*)			
		Lys	NH_3	His	Arg
Standard eluent (pH 6.0 mixed buffer of citrate and phosphate (2:1))	21	22.5	30	39	43.5
	30	24	31	37.5	44.5
pH 6.0, 0.2 *M* Citrate buffer	*ca.* 15	20.5	—	37.5	39
	25	21	28	—	38.5
	30	21.5	28.5	34.5	38.5
pH 6.5, 0.2 *M* Citrate buffer	*ca.* 10	19.5	37	33	34.5
	30	21.5	36	26	35

buffer should be increased more than the standard ratio (citrate: phosphate=2:1, v/v), and then the complete resolution of histidine and arginine will be expected. To make the situation clear, curves plotting the positions at which each amino acid is eluted with mixed buffers of various proportions are given in Fig. 2. Those of the same kind at pH 6.5 are shown in Fig. 3. Some understanding on the effect of pH may also be obtained by comparing both figures.

The only disadvantage in the present method, if any, may be rather troublesome operation required when the resin is buffered. This difficulty comes from the very high buffering capacity of IRC-50 itself, which is in return related to the advantage of the column in its wide adaptability to a sample, *i.e.* a sample may be analyzed without serious difficulties even when the pH, salt concentration and other conditions of its solution are far from optimal.

By the application of this method, the contents of the basic amino acids in the following proteins have been satisfactorily determined; clupeine, salmine, arginine-rich and lysine-rich fractions of calf thymus histone and basic proteins in the premature milt of rainbow trout. The results of these analyses will be reported elsewhere.

SUMMARY

A new chromatographic method was described for the simultaneous

Fig. 2

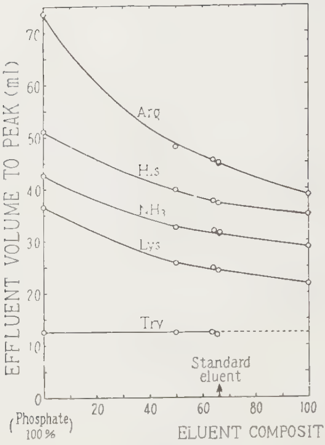

Fig. 3

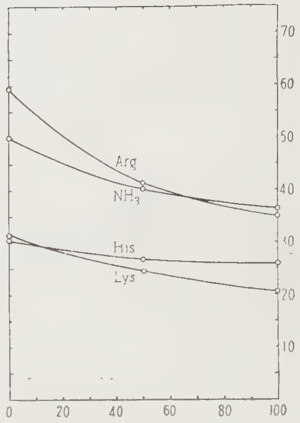

Fig. 2. Effect of eluent composition on effluent volume required untill each peak emerges from a 0.6 × 30 cm. column (volume of resin, 8.5 ml.) at 30°.

The eluent composition is changed in the mixing proportion of 0.2 *M*, pH 6.0 citrate and 0.2 *M*, pH 6.0 phosphate buffer. It is a matter of course that the resin must be previously buffered by each eluent concerned.

Fig. 3. Effect of eluent composition on effluent volume to peak at pH 6.5 and 30°. The citrate and the phosphate buffer mixed in this case are respectively of 0.2 *M* and pH 6.5. Other notes are the same as in Fig. 2.

determination of lysine, histidine and arginine along with ammonia and tryptophan in mixtures of amino acids. The amino acids were separated by elution analysis on a 0.6 × 30 cm. column of Amberlite IRC-50 (XE-64), using a mixture of 2 volumes of citrate buffer and 1 volume of phosphate buffer (0.2 *M*, pH 6.0 in each) as an eluent, and were estimated from the elution curve with recoveries of 100 ± 3 per cent. The procedure is very convenient since a complete resolution of the amino acids is effected with only 50 ml. of the single eluent. Effects of temperature and variations in composition and in pH of the eluent were studied on the position of the emergence of each amino acid, the information from which makes it possible to control the resolution at will.

The author wishes to express his hearty thanks to Prof. T. Ando for his valuable advice and encouragement throughout this investigation and to Mr. T. Tamura for the construction of the fraction collector. The expenses of the present work were aided partly by the Scientific Research Grant given to Prof. T. Ando from the Ministry of Education, to whom the author's thanks are due.

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COMPETITION OF PYRIDINE AND CYANIDE TO HEME AND HEMATIN

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The compounds of heme with various nitrogenous bases as well as CN^- are of great importance for the study of the catalytic function of heme proteins. From this reason, the binding of various basic substances to heme has been studied by many of the investigators (*1*, *2*, *3*). But, on account of the technical difficulties, the more precise investigation, especially, in the competitive binding of pyridine and CN^- to heme (Fe^{II}) or hematin (Fe^{III}), has not yet so far been brought to perfection, since the reaction is essentially of a stepwise process and the property of the intermediary compound can be determined only with difficulties. Only Drabkin (*3*) has reported on the absorption change of hemin solution in the presence of pyridine and CN^-, indicating qualitatively that CN^- combines more easily with hematin (Fe^{III}), while pyridine more easily to heme (Fe^{II}). Mori (*2*) also reported on his semiquantitative studies on the competitive binding of pyridine and CN^- to heme (Fe^{II}) using spectromicroscope and Pulfrich's photometer.

The present authors studied the absorption change in the reaction of heme and hematin with pyridine and CN^- intending to determine the equilibrium constants in the mentioned reaction, and further to determine the absorption figure of the intermediary compound.

EXPERIMENTAL

I. Competitive Binding of Pyridine and Cyanide to Trivalent Hematin-Fe^{III}

(*a*) *Determination of Equilibrium Constants*—Pure hemin crystalls prepared by Willstätter's method (*4*) were dissolved in pyridine water solution, the final concentration of hemin and pyridine being 0.42×10^{-4} *M* and 2.0 *M* respectively. Into a definite volume of this solution, KCN was added in increasing concentrations. By increasing

KCN, the absorption figure of the solution changed from the initial figure of dipyridinehematin-$\mathrm{Fe^{III}}$ $(\mathrm{pyr})_2$ (Fig. 1, I) into that of dicyanhematin-$\mathrm{Fe^{III}}$ $(\mathrm{CN^-})_2$ (Fig. 2, V). Further increase of KCN caused no more change in absorption figure. As shown in the figure of the absorption change (Fig. 1, I–V), an isosbestic point was clearly demonstrated at first at 592 mμ within a definite concentration range of KCN. Above 5×10^{-3} M of KCN, the absorption figure came off from this isosbestic point. But by further increase of KCN, new isosbestic points at 546 and 513 mμ became clearly demonstrable as shown in Fig. 2 (I–V) and the absorption figure approached to that of dicyanhematin.

When, however, hemin was dissolved in KCN solution (their final concentration being 0.42×10^{-5} M and 0.25 M, respectively) and pyridine was increasingly added afterward, the absorption maximum shifted at first toward longer wavelength side and then above 2.0 M of pyridine, the shift occured toward shorter wavelength side. These irregular shifting may be accounted for the solvent effect of pyridine, since, when ethanol or aceton was added instead of pyridine, a similarly irregular shifting of the absorption maximum could be observable. Thus, in the experiment with constant KCN and varying pyridine, the change of the absorption is accounted for the competitive substitution of pyridine with $\mathrm{CN^-}$ and additionally for the solvent effect of pyridine added. In this case, thus, the absorption change can not be accounted for the reaction of an ordinary dissociation equilibrium. In the experiment, however, taking pyridine concentration at constant, the solvent effect of pyridine on the absorption spectrum should be constant. So in the present experiment, the reaction of competing binding of pyridine and $\mathrm{CN^-}$ was studied in the solution of hemin in 2.0 M pyridine by adding $\mathrm{CN^-}$ in varied concentrations.

From Figs. 1 and 2, the reaction could be represented by the following equations in two steps:

$$(1)\quad \mathrm{Hematin\text{-}Fe^{III}}{<}^{\mathrm{pyr}}_{\mathrm{pyr}} + \mathrm{CN^-} \xrightleftharpoons{\mathrm{K_1}} \mathrm{hematin\text{-}Fe^{III}}{<}^{\mathrm{pyr}}_{\mathrm{CN}} + \mathrm{pyr}$$

$$(2)\quad \mathrm{Hematin\text{-}Fe^{III}}{<}^{\mathrm{pyr}}_{\mathrm{CN}} + \mathrm{CN^-} \xrightleftharpoons{\mathrm{K_2}} \mathrm{hematin\text{-}Fe^{III}}{<}^{\mathrm{CN}}_{\mathrm{CN}} + \mathrm{pyr}$$

The process of Reaction (1) was represented by Fig. 1. In the concentration range of KCN of 1×10^{-6} to 5×10^{-5} M, the curves crossed over at a constant isosbestic point indicating the reaction system of two components, namely, hematin-$\mathrm{Fe^{III}}{<}^{\mathrm{pyr}}_{\mathrm{pyr}}$ and hematin-$\mathrm{Fe^{III}}{<}^{\mathrm{pyr}}_{\mathrm{CN}}$.

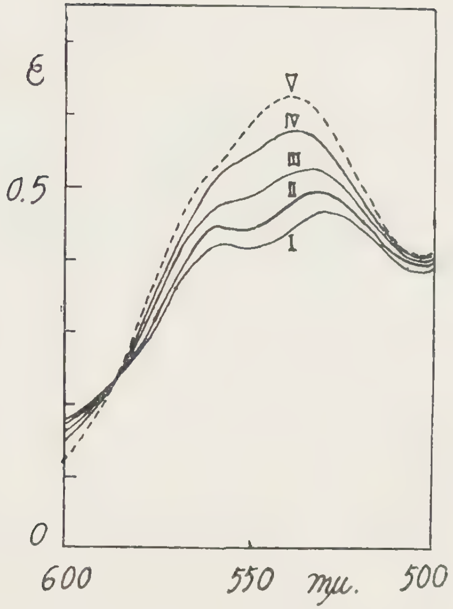


Fig. 1. The absorption change in the reaction of

$$\text{hematin-Fe}^{\text{III}}<^{\text{pyr}}_{\text{pyr}} + \text{CN}^- \rightleftharpoons \text{hematin-Fe}^{\text{III}}<^{\text{pyr}}_{\text{CN}} + \text{pyr}$$

Hemin: 0.42×10^{-4} *M*
KCN: I. 1×10^{-6} *M*. II. 1×10^{-5} *M*. III. 2.5×10^{-5} *M*. IV. 5×10^{-5} *M*. V. 5×10^{-5} *M*

Therefore, in this concentration range of KCN, the relation between the absorption change of the reaction solution at every wavelength against the log concentrations of KCN should be expressed by a sigmoid curve of first order reaction. And when the percentage of $\text{hematin-Fe}^{\text{III}}<^{\text{pyr}}_{\text{CN}}$ formation is expressed by α, the following relation can be introduced.

$$\alpha = \frac{[\text{CN}^-]}{\phi+[\text{CN}^-]}$$

Taking any of the given wavelength, 550 mμ, 530 mμ and 610 mμ for example, the experimentally found values of α were plotted against log concentration of cyanide (Fig. 3, I). Based on these plots, ϕ values

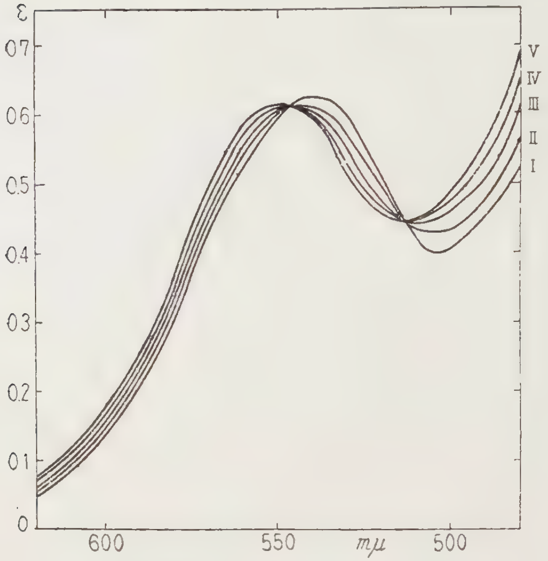
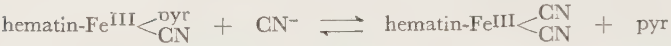


FIG. 2. The absorption change in the reaction of



$$\text{hematin-Fe}^{\text{III}}<^{\text{pyr}}_{\text{CN}} + \text{CN}^- \rightleftharpoons \text{hematin-Fe}^{\text{III}}<^{\text{CN}}_{\text{CN}} + \text{pyr}$$

Hemin: 0.42×10^{-4} *M*

KCN: I. 5×10^{-3} *M*, II. 1×10^{-2} *M*, III. 2.5×10^{-2} *M*., IV. 5×10^{-1} *M*., V. 2.5×10^{-2} *M*.

were calculated and found to be constant: $\phi = 6.31 \times 10^{-5}$ *M*. The limiting KCN concentration of isosbestic range was found to be 5×10^{-5} *M* and at this KCN concentration, a was found to be 41 per cent. Applying $a = \dfrac{[\text{CN}^-]}{\phi + [\text{CN}^-]}$, a theoretical curve was obtained (Fig. 3, I). Experimental values satisfied this theoretical curve.

The process of the Reaction (2) is expressed by Fig. 2. Similarly treating as above, Fig. 3, II was obtained. In this figure also, it was evidenced that the experimental values agreed with theoretical, $\phi = 5.98 \times 10^{-3}$ *M* being computed within the concentration range of KCN in which the formation of dicyanhematin reached above 45 per cent. From the ϕ values obtained in the first and second step, each of the equilibrium constants K_1 and K_2 were calculated as follows:

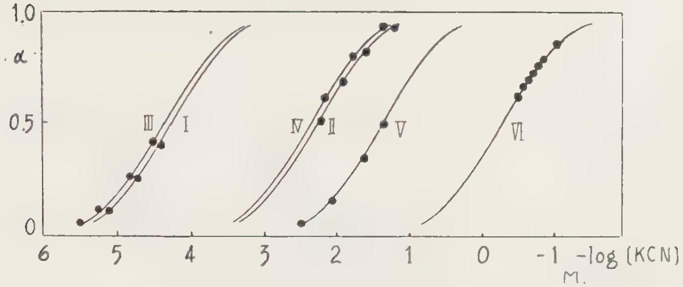


FIG. 3. The relation between the concentration of KCN and α in the substitution reaction.

I. Formation of hematin-$\mathrm{Fe^{III}}{<}\substack{\mathrm{pyr}\\ \mathrm{CN}}$ (from the absorption change in visible range)

II. Formation of hematin-$\mathrm{Fe^{III}}{<}\substack{\mathrm{CN}\\ \mathrm{CN}}$ (from the absorption change in visible range)

III. Formation of hematin-$\mathrm{Fe^{III}}{<}\substack{\mathrm{pyr}\\ \mathrm{CN}}$ (from the absorption change in Soret region)

IV. Formation of hematin $\mathrm{Fe^{III}}{<}\substack{\mathrm{CN}\\ \mathrm{CN}}$ (from the absorption change in Soret region)

V. Formation of heme-$\mathrm{Fe^{II}}{<}\substack{\mathrm{pyr}\\ \mathrm{CN}}$ (from the absorption change in visible and Soret regions)

VI. Formation of heme-$\mathrm{Fe^{II}}{<}\substack{\mathrm{CN}\\ \mathrm{CN}}$ (from the absorption change in visible and Soret regions)

$$K_1 = \frac{\left[\mathrm{Fe^{III}}{<}\substack{\mathrm{pyr}\\ \mathrm{pyr}}\right][\mathrm{CN}]}{\left[\mathrm{Fe^{III}}{<}\substack{\mathrm{pyr}\\ \mathrm{CN}}\right][\mathrm{pyr}]} = 2.10\times10^{-5} = 10^{-4.678} \cdots\cdots\cdots \text{ for the Reaction } (1)$$

$$K_2 = \frac{\left[\mathrm{Fe^{III}}{<}\substack{\mathrm{pyr}\\ \mathrm{CN}}\right][\mathrm{CN}]}{\left[\mathrm{Fe^{III}}{<}\substack{\mathrm{CN}\\ \mathrm{CN}}\right][\mathrm{pyr}]} = 2{\cdot}98\times10^{-3} = 10^{-2.526} \cdots\cdots\cdots \text{ for the Reaction } (2)$$

So far the reaction was studied from the absorption change in the visible range. Following is the result obtained similarly from the investigation at Soret region.

As indicated in Fig. 4, the absorption change in the Soret region was in good accordance with those at the visible range. From Fig. 4,

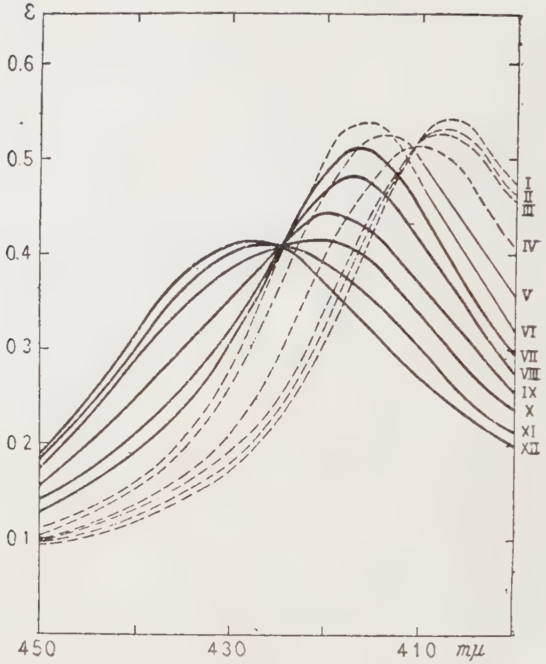


Fig. 4. The absorption change in the reaction of

$$\text{hematin-Fe}^{\text{III}}<^{\text{pyr}}_{\text{pyr}}+\text{CN}^- \rightleftharpoons \text{hematin-Fe}^{\text{III}}<^{\text{pyr}}_{\text{CN}}+\text{pyr} \text{ and}$$

$$\text{hematin-Fe}^{\text{III}}<^{\text{pyr}}_{\text{CN}}+\text{CN}^- \rightleftharpoons \text{hematin-Fe}^{\text{III}}<^{\text{CN}}_{\text{CN}}+\text{pyr}$$

Hemin: 0.4×10^{-5} *M*

KCN: I. 0 *M*, II. 3×10^{-6} *M*, III. 6×10^{-6} *M*, IV. 1.5×10^{-5} *M*, V. 3×10^{-5} *M*, VI. 1×10^{-4} *M*, VII. 3×10^{-3} *M*, VIII. 6×10^{-3} *M*, IX, 1.5×10^{-2} *M*, X. 3×10^{-2}*M*, XI. 6×10^{-2}*M*, XII. 1.5×10^{-1} *M*.

the ϕ values in the reaction of first and second step was obtained on which the theoretical curves were obtained as shown in Fig. 3, III and IV. These curves were satisfied by the experimental values found. K_1 and K_2 were calculated from these ϕ values as follows:

$$K_1 = 2.10\times10^{-5} = 10^{-4.678}$$
$$K_2 = 2.98\times10^{-3} = 10^{-2.526}$$

These K_1 and K_2 values are in good agreement with those obtained from the absorption change in the visible range. Figs. 3, I and III, also II and IV are not precisely the same. This is due to the difference of the concentrations of hemin applied in the experiments in visible range and in Soret region. Disagreement due to the concentration difference of hemin became more significant in the reaction of the first step, concentration of CN^- being very low.

(*b*) *Determination of Absorption Figure of* $Fe^{III}<^{Pyr}_{CN}$, *the Intermediary Compound*—The percentage of the formation of $Fe^{III}<^{pyr}_{CN}$ at each KCN concentration was determined through the *Experiment* (*a*). From the relation between the formation of $Fe^{III}<^{pyr}_{CN}$ in per cent and the corresponding absorption change at each wavelength, the theoretical optical absorption of the pure $Fe^{III}<^{pyr}_{CN}$ at any given wavelength could be obtained by extraporation to 100 per cent of the $Fe^{III}<^{pyr}_{CN}$ formation. By plotting of these optical values, a theoretical absorption figure of $Fe^{III}<^{pyr}_{CN}$ should be given. Applying the method of Kaziro *et al.* (*5*), ε_{mM} was calculatively obtained at each wavelength from which the theoretical figure of hematin-$Fe^{III}<^{pyr}_{CN}$ was obtained as Fig. 5, A, I (visible range) and Fig. 5, C, I (Soret region). The optical figure of the intermediary compound could thus be determined finally, which has so far been remained uncomputible.

II. The Competitive Binding of Pyridine and Cyanide to Bivalent Heme-Fe^{II}

(*a*) *Determination of Equilibrium Constant*—Heme is more easily accessible to molecular aggregation than hematin. In the present experiment, however, base was presented in a high level, so that the molecular aggregation could be excluded.

Hemin was dissolved in pyridine water, the final concentration of hemin and pyridine being 0.28×10^{-4} *M* and 2.0 *M*, respectively. Into a definite volume of this solution, KCN was added in increasing concentrations followed by the reduction with $Na_2S_2O_4$ and the absorption changes were recorded as in Fig. 6. As seen in Fig. 6 the pyridine hemochrome figure changed gradually into the figure of heme-$Fe^{II}<^{pyr}_{CN}$

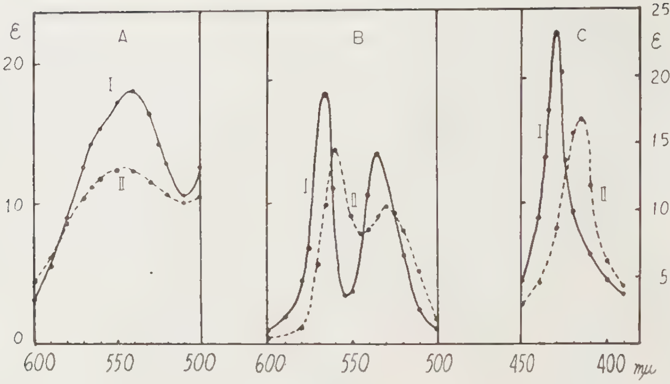


FIG. 5. The absorption figure of $\mathrm{Fe}<^{\mathrm{pyr}}_{\mathrm{CN}}$ in heme and hematin calculatively obtained.

A. I. hematin-$\mathrm{Fe}^{\mathrm{III}}<^{\mathrm{pyr}}_{\mathrm{CN}}$

II. mathematical mean of the absorptions of hematin-$\mathrm{Fe}^{\mathrm{III}}<^{\mathrm{pyr}}_{\mathrm{pyr}}$ and hematin-$\mathrm{Fe}^{\mathrm{III}}<^{\mathrm{CN}}_{\mathrm{CN}}$

B. I. heme-$\mathrm{Fe}^{\mathrm{II}}<^{\mathrm{pyr}}_{\mathrm{CN}}$

II. mathematical mean of the absorptions of heme-$\mathrm{Fe}^{\mathrm{II}}<^{\mathrm{pyr}}_{\mathrm{pyr}}$ and heme-$\mathrm{Fe}^{\mathrm{II}}<^{\mathrm{CN}}_{\mathrm{CN}}$

C. I. hematin-$\mathrm{Fe}^{\mathrm{III}}<^{\mathrm{pyr}}_{\mathrm{CN}}$

II. heme-$\mathrm{Fe}^{\mathrm{II}}<^{\mathrm{pyr}}_{\mathrm{CN}}$

with increasing concentration of KCN, demonstrating three isosbestic points at 528, 544.5 and 562 mμ. Above a definite concentration of KCN, the absorption change came off from the isosbestic change. Thus, the reaction of this series is also a stepwise process as expressed by the following equations:

$$(1)\quad \mathrm{Fe}^{\mathrm{II}}<^{\mathrm{pyr}}_{\mathrm{pyr}} + \mathrm{CN}^- \underset{}{\overset{K_3}{\rightleftarrows}} \mathrm{Fe}^{\mathrm{II}}<^{\mathrm{pyr}}_{\mathrm{CN}} + \mathrm{pyr}$$

$$(2)\quad \mathrm{Fe}^{\mathrm{II}}<^{\mathrm{pyr}}_{\mathrm{CN}} + \mathrm{CN}^- \underset{}{\overset{K_4}{\rightleftarrows}} \mathrm{Fe}^{\mathrm{II}}<^{\mathrm{CN}}_{\mathrm{CN}} + \mathrm{pyr}$$

The ϕ value in the first reaction was found to be $4.68 \times 10^{-2}\ M$,

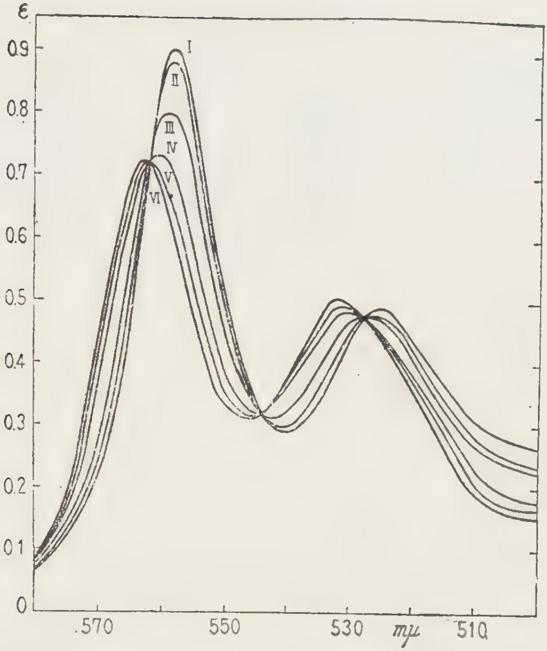
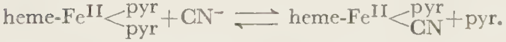


FIG. 6. Absorption change in the reaction of



$$\text{heme-Fe}^{\text{II}}<^{\text{pyr}}_{\text{pyr}}+\text{CN}^{-}\rightleftharpoons\text{heme-Fe}^{\text{II}}<^{\text{pyr}}_{\text{CN}}+\text{pyr}.$$

Hemin: $0.28\times10^{-4}\ M$

KCN: I. $0\ M$, II. $5\times10^{-2}\ M$, III. $1\times10^{-2}\ M$, IV. $2.5\times10^{-2}\ M$, V. $5\times10^{-2}\ M$, VI. $1\times10^{-1}\ M$.

obtained by the procedure similar to that in the *Experiment* I. Applying this ϕ value, a theoretical curve of first order reaction was obtained as shown in Fig. 3, V. The curve was satisfied by the experimental values found within the concentration range of KCN leading to the formation of $\text{Fe}^{\text{II}}<^{\text{pyr}}_{\text{CN}}$ up to 50 per cent.

The same reaction was studied by the absorption change in the Soret region. The result agreed fairly well with the above observation. An isosbestic point was found at 423 mμ. The value of ϕ was found in this case to be the same as above. From this, K_3 was calculated as follows.

$$K_3=\frac{\left[\mathrm{Fe^{II}}<^{\mathrm{pyr}}_{\mathrm{pyr}}\right][\mathrm{CN^-}]}{\left[\mathrm{Fe^{II}}<^{\mathrm{pyr}}_{\mathrm{CN}}\right][\mathrm{pyr}]}=2.34\times10^{-2}=10^{-1.630}$$

As the next experiment, hemin was dissolved in KCN solution of a definite concentration and the concentration of pyridine was varied. The absorption change proceeded as shown in Fig. 7. As mentioned already, in the experiments with hematin $\mathrm{Fe^{III}}$, solvent effect of pyridine

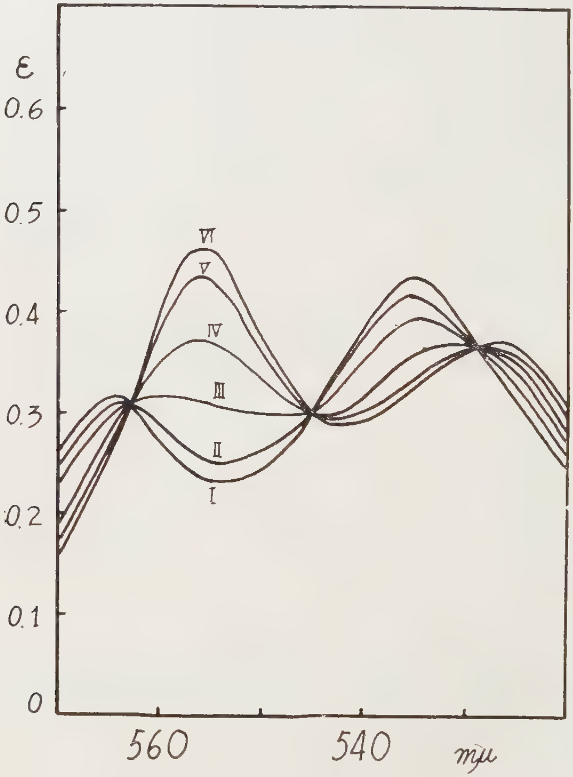


Fig. 7. Absorption change in the reaction of

$$\text{heme-}\mathrm{Fe^{II}}<^{\mathrm{pyr}}_{\mathrm{CN}}+\mathrm{CN^-}\rightleftharpoons\text{heme-}\mathrm{Fe^{II}}<^{\mathrm{CN}}_{\mathrm{CN}}+\mathrm{pyr}$$

Hemin: 0.28×10^{-4} *M*

Pyridine: I. 0 *M*, II. 1×10^{-2} *M*, III. 5×10^{-2} *M*, IV. 1.25×10^{-1} *M*, V. 2×10^{-1} *M*, VI. 2.5×10^{-1} *M*.

upon the absorption change was significantly strong. In this case, namely of bivalent Fe^{II}, however, the binding affinity of pyridine to heme-Fe^{II} is strongly enough to allow the observation of absorption change in more low concentration range of pyridine without any disturbance of solvent effect upon absorption. Thus, from Fig. 7, ϕ value was calculated similarlyl to the above mentioned procedure in *Experiment* (*a*) based on the equation :

$$\alpha = \frac{[\mathrm{pyr}]}{\phi + [\mathrm{pyr}]}$$

A theoretical curve was obtained based on this ϕ value as shown in Fig. 8. The experimental values found satisfy the theoretical curve. Similar absorption change was also recorded in the Soret region. Under the definite concentration range of pyridine an isosbestic point was demonstrated at 428.5 mμ, the reaction proceeding toward $Fe^{II}<^{\mathrm{pyr}}_{\mathrm{CN}}$. The ϕ value found in this experiment agreeded well with that obtained by the observation in the visible range. Based on this ϕ value a theoretical dissociation curve was established corresponding to the absorption change by the change of KCN concentration with constant pyridine concentration (2.0 *M*) as indicated in Fig. 3, VI. K_4 was calculated based on this ϕ value as follows :

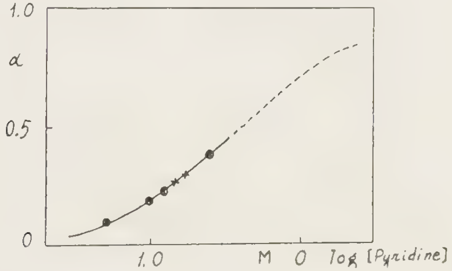


FIG. 8. The relation between the concentrations of pyridine and α in the reaction of heme-$Fe^{II}<^{\mathrm{CN}}_{\mathrm{CN}} + \mathrm{pyr} \rightleftharpoons$ heme-$Fe^{II}<^{\mathrm{pyr}}_{\mathrm{CN}} + CN^-$.

—×—×— from the observation in visible range
—○—○— from the observation in Soret region

$$K_4 = \frac{\left[\mathrm{Fe^{II}}{<}^{\mathrm{CN}}_{\mathrm{CN}}\right][\mathrm{pyr}]}{\left[\mathrm{Fe^{II}}{<}^{\mathrm{pyr}}_{\mathrm{CN}}\right][\mathrm{CN^-}]} = 1.76 = 10^{0.512}$$

(*b*) *Determination of the Absorption Figure of the Intermediary Compound, Heme-*$\mathit{Fe^{II}}{<}^{Pyr}_{CN}$—By the use of procedure described in the *Experiment I*, (*b*), a theoretical absorption figure of heme-$\mathrm{Fe^{II}}{<}^{\mathrm{pyr}}_{\mathrm{CN}}$ in its pure state was determined as shown in Fig. 5, B, I (visible range) and C, II (Soret region).

DISCUSSION AND SUMMARY

The competitive binding of pyridine and $\mathrm{CN^-}$ to heme and hematin respectively was quantitatively studied through the absorption change in the visible as well as in the Soret region of the spectrum by means of electric spectrophotometer. The following results were obtained.

1. Both in heme and hematin the substitution reaction of pyridine to $\mathrm{CN^-}$ proceeded in two steps of typically consecutive reaction : $\mathrm{Fe}{<}^{\mathrm{pyr}}_{\mathrm{pyr}} \rightarrow \mathrm{Fe}{<}^{\mathrm{pyr}}_{\mathrm{CN}} \rightarrow \mathrm{Fe}{<}^{\mathrm{CN}}_{\mathrm{CN}}$. This is suggesting that the bonding of pyridine and $\mathrm{CN^-}$ to Fe seems to be of the same nature. Their binding to heme and hematin has been well assumed to be coordinative and the ionic bonding of $\mathrm{CN^-}$ seems to be unprobable though in monocyanoferriprotoporphyrin an ionic bonding of $\mathrm{CN^-}$ may also be suggested to be possible. But if the bonding of $\mathrm{CN^-}$ in $\mathrm{Fe^{II}}{<}^{\mathrm{CN}}_{\mathrm{CN}}$ is ionic, the compound should have two negative charges.

The equilibrium constants in every reaction steps were determined as follows :

(i) $\text{Hematin-Fe}^{\mathrm{III}}{<}^{\mathrm{pyr}}_{\mathrm{pyr}} + \mathrm{CN^-} \overset{K_1}{\rightleftarrows} \text{hematin-Fe}^{\mathrm{III}}{<}^{\mathrm{pyr}}_{\mathrm{CN}} + \text{pyr.}$

$$K_1 = 10^{-4.678}$$

(ii) $\text{Hematin-Fe}^{\mathrm{III}}{<}^{\mathrm{pyr}}_{\mathrm{CN}} + \mathrm{CN^-} \overset{K_2}{\rightleftarrows} \text{hematin-Fe}^{\mathrm{III}}{<}^{\mathrm{CN}}_{\mathrm{CN}} + \text{pyr.}$

$$K_2 = 10^{-2.526}$$

(iii) $\text{Heme-Fe}^{\mathrm{II}}{<}^{\mathrm{pyr}}_{\mathrm{pyr}} + \mathrm{CN^-} \overset{K_3}{\rightleftarrows} \text{heme-Fe}^{\mathrm{II}}{<}^{\mathrm{pyr}}_{\mathrm{CN}} + \text{pyr.}$

$$K_3 = 10^{-1.630}$$

$$\text{(iv)}\quad \text{Heme-Fe}^{\text{II}}<^{\text{pyr}}_{\text{CN}} + \text{CN}^- \overset{K_4}{\rightleftharpoons} \text{heme-Fe}^{\text{II}}<^{\text{CN}}_{\text{CN}} + \text{pyr.}$$

$$K_4 = 10^{0.512}$$

The results are indicating that pyridine combines more easily to heme-Fe^{II} than to hematin-Fe^{III}, while CN^- more easily to hematin-Fe^{III} than to heme-Fe^{II}. The difference of the pK′ values of the first and the second step in both of the reaction of heme and hematin were determined as 2.142 and 2.152 respectively. This means that the second step beginns after that the formation of $\text{Fe}<^{\text{pyr}}_{\text{CN}}$ reached up to 50 per cent in the first step reaction. The dissociation curves of the first and the second step make a continuous curve without any flexion, indicating that the substitution occurs simply following the mass law of the reaction.

2. The ideal absorption figures of the intermediary compounds in their pure state,

$$\text{Heme-Fe}^{\text{II}}<^{\text{pyr}}_{\text{CN}} \text{ and hematin-Fe}^{\text{III}}<^{\text{pyr}}_{\text{CN}}$$

could be determined based on the experimental data obtained. Their individual figures are not the mathematical means of the absorptions of both of the limiting figures $\text{Fe}<^{\text{pyr}}_{\text{pyr}}$ and $\text{Fe}<^{\text{CN}}_{\text{CN}}$; they are indicated by more specific characterisation of the absorption.

A similar series of experiment was reported by Horiguchi (*6*) in our laboratory dealt with the competitive binding of alkali denatured globin and CN^- to heme-Fe. It was noted in his experiment also that the absorption figure of heme-$\text{Fe}^{\text{II}}<^{\text{gl}}_{\text{CN}}$ was not indicated by the mathematical mean of the absorptions of heme-$\text{Fe}^{\text{II}}<^{\text{gl}}_{\text{gl}}$ and that of $\text{Fe}^{\text{II}}<^{\text{CN}}_{\text{CN}}$, but it was more specific.

3. Both in heme and hematin, the affinity of pyridine to Fe seems to be somewhat strengthened in the intermediary compound $\text{Fe}<^{\text{pyr}}_{\text{CN}}$ than in $\text{Fe}<^{\text{pyr}}_{\text{pyr}}$. As already mentioned, the difference of the pK values in the first and second steps were found to be 2.142 and 2.152. The difference of the pK values, however, are not exactly 2.0, but they were obtained somewhat in higher value. This is indicating that the affinity of pyridine to Fe is somewhat intensified in the intermediary

compound of $\mathrm{Fe}<^{\mathrm{pyr}}_{\mathrm{CN}}$.

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STUDIES ON THE OXIDATION MECHANISM OF METHYL GROUP

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As Asano (*1*), Hirohata and their co-wokers (*2, 3*) have shown, L-rhodinic acid and the related compounds are subjected to ω-oxidation to a large extent (more than 25 per cent) in the animal organism, one of the methyl groups at the ω-position being oxidised to carboxyl group.

The mechanism of the oxidation, however, remains yet indistinct, though it is presumed that it proceeds over alcohol-and aldehyde formation.

In the present work toluene was chosen as the substrate instead of rhodinic acid, as its alcohol and aldehyde analogues are well known, and as the conversion of toluene to benzoic acid in animal organisms is quite evident since the classic report of Schultzen and Naunyn (*4*). Wagner (*5*) and Tausson (*6*) reported previously that toluene was readily utilized by certain bacteria as the sole source of energy for aerobic growth in an inorganic medium. The steps of the oxidation could be satisfactorily pursued by means of adaptive enzymes produced in *Pseudomonas aeruginosa*.

Moreover, the oxidation of toluene by animal tissue was also investigated.

EXPERIMENTAL AND RESULTS

Cultivation—A strain of *Pseudomonas aeruginosa** was used throughout the present experiments.

The bacteria were cultivated at 38° for 20 to 40 hours. The culture media composed as follows were autoclaved after the adjustment of pH to 7.0.

Peptone 1.00 per cent; beef extract 1.00 per cent; NaCl 0.50

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* This strain was kindly given by Dr. Y. Jinnaka, (Department of Bacteriology, Faculty of Medicine, Kyushu University).

per cent.

Cell suspensions—The bacteria thus cultured were harvested by centrifugation, washed twice with sterile distilled water and finally suspended in $M/15$ phosphate buffer of pH 7.0. The total nitrogen of cells in the suspension was about 1 mg./ml.

For starvation of the cells, the cell suspension was placed in an ice-box over night or incubated for 3 hours at 38°.

Substrate—Toluene, benzyl alcohol and benzaldehyde were purified by conventional method. Toluene was used in its saturated aqueous solution and benzyl alcohol, benzaldehyde, benzoic acid (as sodium salt, pH adjusted to 7.0) and pyrocatechine in 0.01 M solution.

Preparation of Bacteria Adapted to Each Substrate—In the case of toluene, an Erlenmyer flask with a centre well such as in a Warburg vessel was used. The cell suspension in $M/15$ phosphate buffer saturated with toluene were put in the main compartment and toluene was dropped into the centre well and the flask was closed tightly with the glass stopper. After cell suspensions were kept at 25° for 10 hours, they were centrifuged, washed twice with distilled water and suspended in $M/15$ phosphate buffer.

In order to obtain bacteria adapted to benzyl alcohol, benzaldehyde and benzoic acid, respectively, the cell suspensions were incubated for 2 hours at 38° in the presence of each substrate (concentration 0.001 M). After incubation, the cells were centrifuged, washed twice with distilled water, and suspended in $M/15$ phosphate buffer.

Behavior of Various Cell Suspension Preparations to Substrate—The degree of adaptation was determined manometrically by the oxygen uptake with a Warburg manometer after addition of the substrate to each cell suspension. The experiments were carried out with 1 ml. of cell suspensions and 0.2 ml. of 0.01 M substrate at 38° in an atmosphere of air.

The suspended cells not previously treated with the substrate hardly oxidised benzyl alcohol, benzaldehyde, benzoate and pyrocatechine in the first 60 minutes: their oxygen uptake remained at the autorespiratory rate for about 60 minutes following the addition of the substrate, and then increased exponentially to a steady maximum rate which was maintained till the substrate exhaution (Fig. 1).

On the contrary, the cells previously treated with toluene oxidised benzyl alcohol, benzaldehyde, benzoate and pyrocatechine without any lag phase (Fig. 2). But hydroquinone, salicylic acid, phenol, *p*-toluene sulfonic acid and cresol were not oxidised.

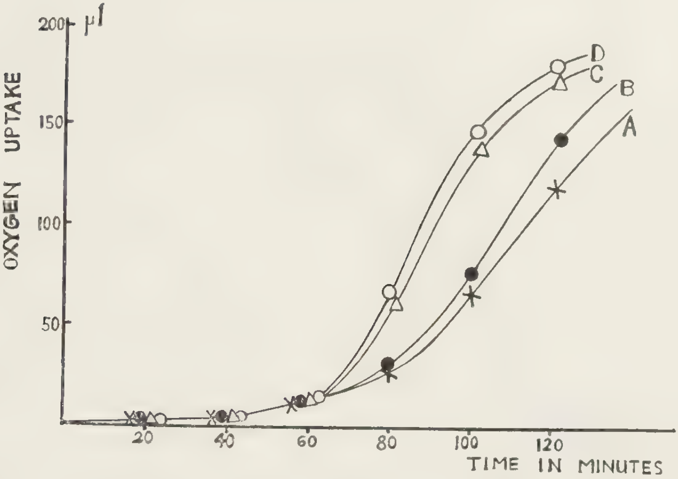

FIG. 1. Oxygen uptake by *Pseudomonas aeruginosa* cultivated on basis media with 2 μM of various substrates.

Experiments were generally carried out with 1 ml. of cell suspension (*M*/15 phosphate buffer pH 7.0) and 0.2 ml. of *M*/100 substrate in a Warburg apparatus at 30° in an atomosphere of air. The total volume of reaction mixture was always made to 2 ml. Autorespiration was subtracted from each value.

A: Benzyl alcohol, B: Benzaldehyde, C: Benzoate, D: Pyrocatechine.

Furthermore, the cells treated with benzyl alcohol oxidised benzaldehyde, benzoate and pyrocatechine without any lag phase (Fig. 3), while those treated with benzaldehyde oxidised benzoate and pyrocatechine without any lag phase, but did not respond to benzyl alcohol immediately (Fig. 4). In this case, the lag phase for alcohol oxidation was shorter than that of those not previously treated with substrate. The cells treated with benzoate oxidised pyrocatechine without any lag phase but did not oxidise benzyl alcohol and benzaldehyde immediately (Fig. 5).

Acetone-dried Cells—The cells adpated to toluene were dried with acetone and 50 mg. of the dried powder was suspended in 10 ml. of *M*/15 phosphate buffer. With 1 ml. of the suspension, oxygen consumption for each substrate was measured, and found to be active only for pyrocatechine (Fig. 6), but not for benzyl alcohol, benzaldehyde and

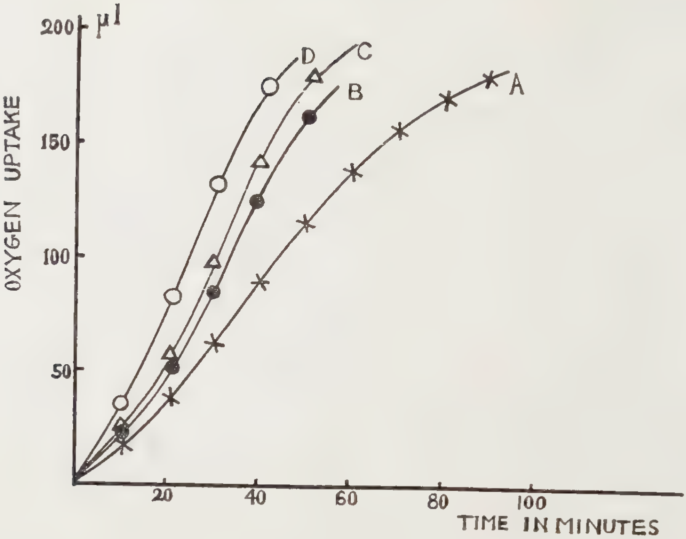

Fig. 2. Oxygen uptake by *Pseudomonas aeruginosa* adapted to toluene with 2 μM of various substrates.

Experiments conditions were similar to those shown in Fig. 1. except using cells adapted to toluene.

A: Benzyl alcohol, B: Benzaldehyde, C: Benzoate, D: Pyrocatechine.

benzoate. On the other hand, the acetone-dried cells unadapted to toluene were inactive for all substrates.

Oxidation Products of Toluene—A spectrophotometric method was applied to the investigation of toluene oxidation products. The above mentioned Erlenmyer flask with a centre well was employed again in the same way. Reaction mixture was incubated at 25°, and 2 ml. of it was pipetted at interval of 30 minutes in a glass tube with stopper, added with 1 ml. of $2N$ H_2SO_4 and shaken with 5 ml. of ether for 3 minutes. The optical density of the ether layer was measured spectrophotometrically (Beckman-Model-DU) at 257 mμ and 243 mμ (Fig. 7). The wave length of the maximum absorption was found to be 262 mμ for toluene, 257 mμ for benzyl alcohol and 243 mμ for benzaldehyde in ether solution. As shown in Fig. 7, the optical density at 257 mμ and 243 mμ increased with the progress of the reaction.

In the above experiment, the reaction mixture incubated for 2

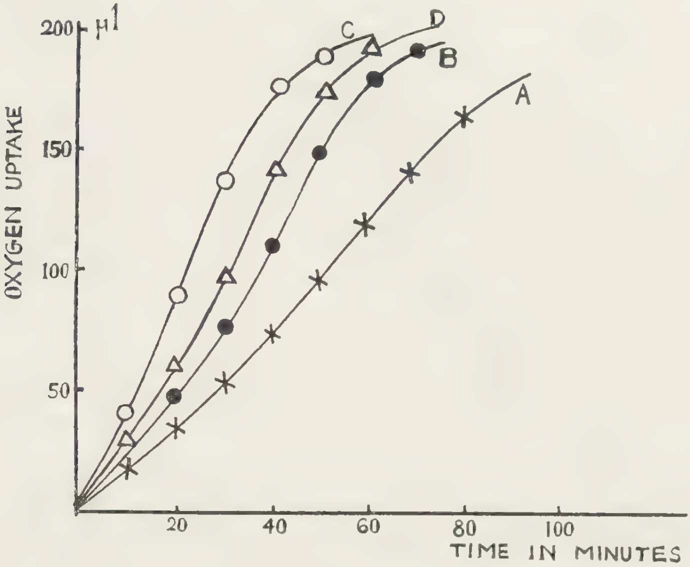

Fig. 3. Oxygen uptake by *Pseudomonas aeruginosa* adapted to benzyl alcohol with 2 μM of various substrates.

Experiments conditions were similar to those shown in Fig. 1. except using cells adapted to benzyl alcohol.

A: Benzyl alcohol, B: Benzaldhyde, C: Benzoate, D: Pyrocatechine.

hours was centrifuged, and ammoniacal solution of silver nitrate was added to the supernatant liquid. At room temperature the supernatant became dark brown. Under the same condition, even 10^{-6} M solution of pyrocatechine became dark brown after the addition of ammoniacal solution of silver nitrate.

Dehydrogenation Test—A Thunberg tube was employed throughout this experiments. First, 1 ml. of freshly prepared 10^{-3} M triphenyltetrazolium chloride (T.T.C.) and 0.4 ml. of 10^{-2} M substrate solution were put in its side arm. In the case of toluene test, however, 1 ml. of its saturated solution and in that of blind test, 1 ml. of distilled water, was added. In each experiment 2 ml. of cell suspensions (adapted to toluene) was placed in the tube. The final volume of reaction mixture was always made to 5 ml. The Thunberg tube containing the reaction mixture was evacuated to 20 mm.-Hg in 3 minutes and set in a water

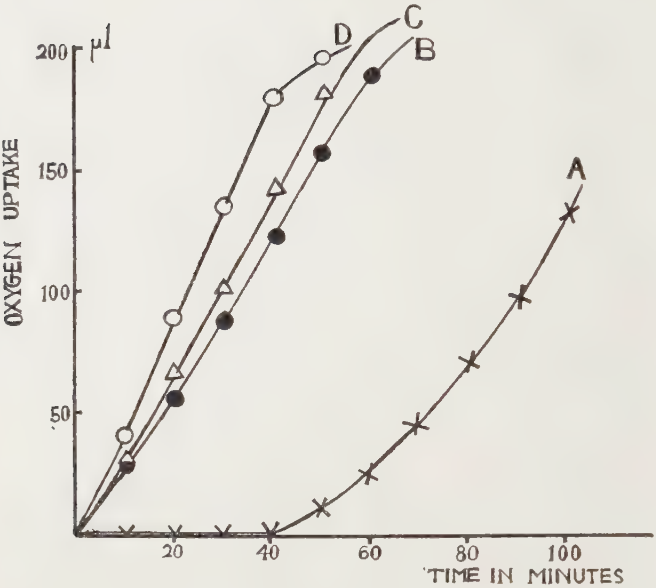

FIG. 4. Oxygen uptake by *Pseudomonas aeruginosa* adapted to benzaldehyde with 2 μM of various substrates.

Experiments conditions were similar to those shown in Fig. 1. except using cells adapted to benzaldehyde.

A: Benzyl alcohol, B: Benzaldehyde, C: Benzoate, D: Pyrocatechine.

bath (38°), the substrate and T.T.C. in the side arm were tipped into the tube; the reaction was allowed to proceed for 30 minutes, and was stopped by the addition of 2 ml. of 20 per cent trichloroacetic acid into each tube. The mixture was added with 5 ml. of ethylacetate, shaken vigorously and centrifuged. The color density of the ethylacetate layer was measured photometrically and the formazan produced was calculated.

Along with this determination, the decolorization time of methylene blue was estimated with 1 ml. of 4×10^{-4} M M. B. in place of T.T.C. under otherwise same conditions of the reaction. The results of the two experiments are shown in Table I.

Animal Experiment—Rabbits and rats were employed. The liver was homogenized in ice-cold Krebs-Ringer solution of pH 7.2 (1 :3); one drop of toluene (about 5 mg.) was added to 15 ml. of the homoge-

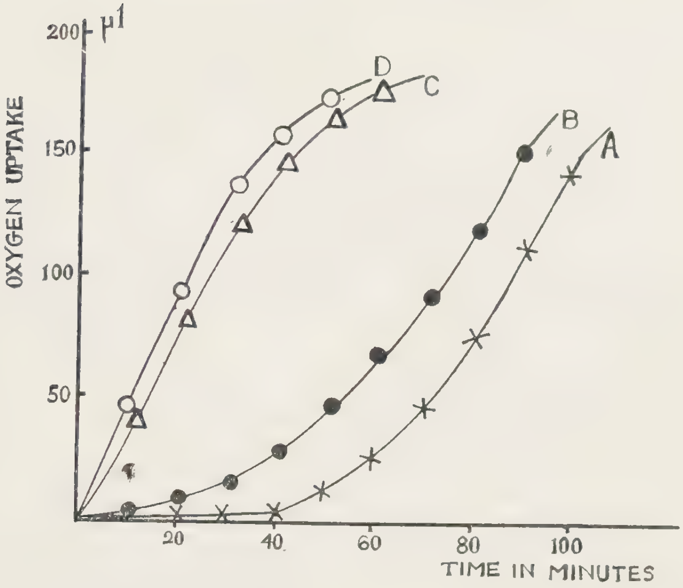

FIG. 5. Oxygen uptake by *Pseudomonas aeruginosa* adapted to benzoic acid with 2 μM of various substrates.

Experiments conditions were similar to those shown in Fig. 1. except using cells adpated to benzoate.

A: Benzyl alcohol, B: Benzaldehyde, C: Benzoate, D: Pyrocatechine.

nate, and the reaction mixture was incubated at 30° for 1 hour, and was deproteinized by the method of Folin-Wu; the filtrate was extracted twice with ether. The ether was evaporated at low temperature as possible. The residue was dissolved in a small amount of ether, and chromatographed by the method of Kalbe (*7*). The results are given in Table II. The residue was further treated with *p*-nitrobenzoylchloride after Holley's method (*8*) to esterify the benzyl alcohol anticipated to the nitrobenzoate. The sample obtained was chromatographed by the method of Rice *et al.* (*9*). The results are shown in Table III.

DISCUSSION

Suda (*10*) reported previously that if bacteria were adapted to the substance of higher metabolic order, the adaptive enzymes for substances

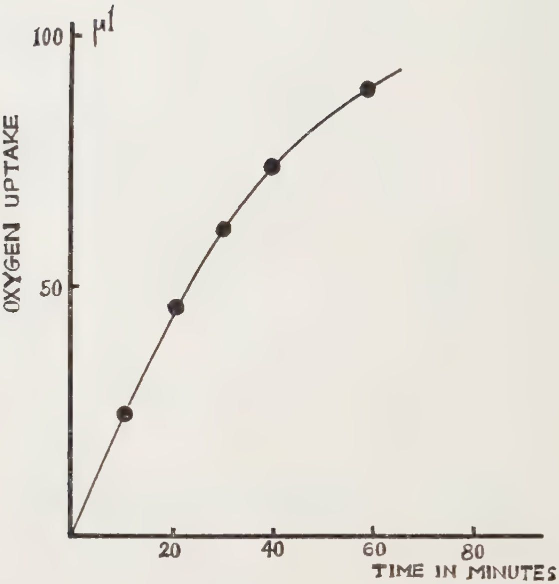

FIG. 6. Oxydation of pyrocatechine by aceton dried cells adapted to toluene.

Using 1 ml. of aceton dried cells suspensions adapted to toluene (50 mg. in *M*/15 phosphate buffer pH 7.2) and 0.4 ml. of *M*/100 pyrocatechine, oxygen consumption were measured in a Warburg apparatus at 38° in an atomosphere of air. Autorespiration was subtracted from measured value.

that came below in the metabolic pathway might also be produced and conversely if the bacteria were adapted to a substance of lower metabolic order, the enzymes for substances of higher order might not be produced. The available evidence in the present experiments (see Fig. 1 to 5) shows the possibility that toluene may be oxidised via benzyl alcohol, benzaldehyde, benzoate and pyrocatechine.

In fact, the formation of pyrocatechine is demonstrated at the incubation of toluene with the suspension of living cells, while acetone-dried cells adapted to toluene take up so much oxygen that pyrocatechine can be further oxidized, probably due to the formation of pyrocatecase in the cells, an enzyme stable to acetone as Suda has already confirmed.

Spectrophotometric investigation demonstrates also the possibility

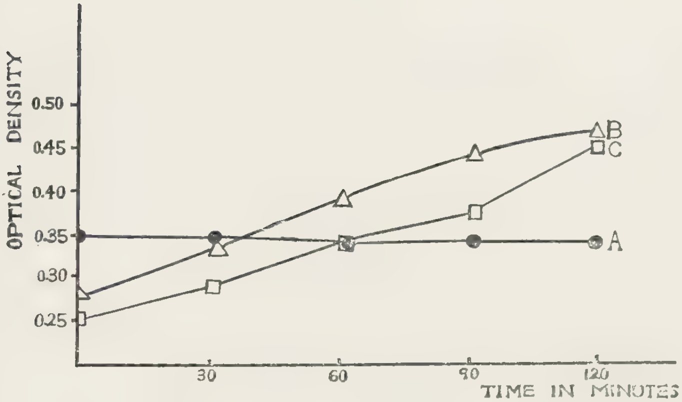

FIG. 7. Spectrophotometric investigation of toluene oxidation product by *Pseudomonas aeruginosa.*

Cells adapted to toluene were incubated in $M/15$ phosphate buffer saturated with toluene at 25° using special flask (see text). 2 ml. of reaction mixture was extracted with 5 ml. of ether with interval of 30 minutes. The optical density of ether layer was measured spectrophotometrically (Beckman Model DU) at 257 mμ (Curve B) and 243 mμ (Curve C). Also, using boiled cell suspension, absorption at 262 mμ (Curve A) was measured.

TABLE I

Dehydrogenation Test by Pseudomonas aeruginosa Adapted to Toluene

Substrate	Formation of formazan	Decolorization time of M. B.
	μM	*minutes*
Control	0.26	28
Toluene	0.32	19
Benzyl alcohol	0.39	17
Benzaldehyde	0.47	13

2 ml. of cell suspension (adapted to toluene), 0.4 ml. of 10^{-2} M substrate (in the case of toluene, 1 ml. of its saturated solution) and 1 ml. of 10^{-3} M T.T.C. were incubated in a Thunberg tube anaerobically at 38° for 30 minutes. Formazan produced was extracted with 5 ml. of ethyl acetate and measured photometrically.

Decolorization time of methylene blue was measured using 1 ml. of its 4×10^{-4} M solution instead of T.T.C.

TABLE II

Paper Chromatographic Analysis

Solvent	Rf value	
	Benzoic acid	Sample
Isopropanol-ammonium carbonate buffer (3:1, *v/v*)	0.62	0.62
Pyridine-water (80:20, *v/v*)	0.66	0.65

TABLE III

Paper Chromatographic Analysis

Solvent	Rf value	
	Nitrobenzoate of benzyl alcohol	Sample
Petroleum ether-methanol (98:2, *v/v*)	0.88	0.87
Pyridine-water (30:90, *v/v*)	0.84	0.85

of the formation of benzyl alcohol and benzaldehyde from toluene.

Atchley (*11*) postulated that the intermediate products of the oxidation of isobutyric acid comprised hydroxyisobutyric acid, methylacrylic acid and methylmalonic semialdehyde. But he did not pursue the oxidation mechanism. As shown in Table I. dehydrogenation is more significant in the presence of a substrate than in its absence. However, with all such evidences it is difficult to determine whether the conversion from toluene to benzyl alcohol is induced by dehydrogenation anaerobically or otherwise. It may be safe to consider, however, that benzyl alcohol and benzaldehyde are dehydrogenated by such mechanism as an alcohol- and aldehyde-dehydrogenase action.

Also in the toluene oxidation by a mammalian liver, benzyl alcohol and benzoic acid were identified by paper chromatography though the formation of benzaldehyde could not be demonstrated probably owing to its oxidation during the chromatographic procedures.

SUMMARY

Toluene was chosen as substrate in investigation of the oxidation

mechanism of the methyl group. Succesive adaptation by a strain of *Pseudomonas aeruginosa*, was utilized for the analysis of the oxidation process of toluene. The results showed the possibility that toluene was oxidised via benzyl alcohol, benzaldehyde and benzoic acid. The formation of the former two and pyrocatechine (from toluene) was demonstrated.

In mammalian livers, benzyl alcohol and benzoic acid were identified as products of toluene oxidation.

The author wishes to thank Prof. Dr. R. Hirohata for his guidance in carrying out this work. This work was carried out partly by the aid of the Scientific Research Funds of the Ministry of Education.

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The Journal of Biochemistry, Vol. 43, No. 4, 1956

ON THE MECHANISM OF THE ACTIVATION OF α-CHYMOTRYPSINOGEN TO α-CHYMOTRYPSIN

IV. THE PEPTIDE LIBERATION DURING ACTIVATION*

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In the previous work (*1*), formation of several intermediate proteins had been recognized electrophoretically during the slow activation process of α-chymotrypsinogen to α-chymotrypsin, and later it was confirmed by chromatographic analysis on columns of ion-exchange resin, Dowex 50 (*2*) and *N*-terminal-group analysis (*3*). It was thus concluded by the author that the conversion of α-chymotrypsinogen to α-chymotrypsin is not a simple process but a highly complex one.

In the previous communication (*4*), an attempt to prepare crystalline α-chymotrypsin by the slow activation under dialysis led the author to isolate a new crystalline protein, which was identical with none of the known crystalline chymotrypsins, as far as crystalline form concerned, and was supposed to be a new crystalline chymotrypsin.

In the present work the slow activation under dialysis also made to isolate the liberated peptides without further non-specific hydrolysis at several intermediate stages of the activation, and the constituent amino acid residues of the liberated peptides were investigated by two-dimension paper chromatographic technique. At the same time, the free peptides which were liberated during the slow activation without dialysis, were converted into dinitrophenyl(DNP)-derivatives, and the nature of them were studied by paper ionophoretic technique and paper chromatographic one.

As the results of the present experimental studies, which dealt in particular with the nature of the liberated peptides, it was shown that the slow activation of α-chymotrypsinogen was accompanied by the formation of several peptides, two of which were identified to be seryl-arginine and threonyl-aspartic acid, and which probably arise from one or two common precursor composed of several amino acid residues.

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EXPERIMENTAL

α-Chymotrypsinogen—α-Chymotrypsinogen was prepared from fresh beef pancreas by the method of Kunitz *et al.* (*5*). This was crystallized thrice with ammonium sulfate, and then once with aqueous ethanol.

Trypsin—Trypsin was prepared from the mother liquor of crystalline α-chymotrypsinogen by the method of Kunitz *et al.* (*6*).

Slow Activation of α-Chymotrypsinogen under Dialysis—Crystalline salt-free α-chymotrypsinogen (1.52 g.) was suspended in 10 ml. of distilled water and dissolved by the addition of several drops of 5 *N* sulfuric acid, and then 4.2 ml. of *M*/2 phosphate buffer (pH 7.6) and a quantity of 5 *N* sodium hydroxide equivalent to the acid were added. The mixture was poured into a cellophane bag, washed in with 1.5 ml. of distilled water, and dialyzed at 5° against 20 ml. of *M*/8 phosphate buffer (pH 7.6). Then the mixture was added with 0.21 mg. crystalline trypsin dissolved in 0.85 ml. of 0.0025 *N* hydrochloric acid and activated for 48 hours under dialysis at 5°. The dialyzing buffer was replaced every 10 hours by a new buffer which was cooled to 5° before use.

The 0.1 ml. aliquots of the activation mixture were removed at various time intervals and their proteolytic activity were determined spectrophotometrically by the method described in the previous report (*1*), and the activity was expressed as per cent of the maximum activity which was attained by 48 hours activation (Fig. 1).

Every dialyzing buffer which was replaced every 10 hours, was instantly lyophilized and analyzed as follows. A 30 mg. aliquot of the lyophilized substance was dissolved in 1 to 2 ml. of distilled water, adjusted to about pH 5.0 with *N* hydrochloric acid and made to 5 ml. with distilled water. Then the ninhydrin colour value of the solution was determined photometrically according to the method described by Stein and Moore (7), and the result was shown in Fig. 1.

In order to desalt, the lyophilized substance was subjected to one-dimension paper chromatography on a wide filter paper with phenol-water (9:1) as solvent. After about 18 hours developement, the peptide was separated as a wide spreaded zone (Rf 0.3∼0.9), although the contaminated inorganic salt still remained near the original line. The peptide zone was then cut, eluted with water, and concentrated *in vacuo*. It was then repeatedly subjected to two-dimension paper chromatography with *n*-butanol-acetic acid-water (4:1:1) and phenol-water (9:1).

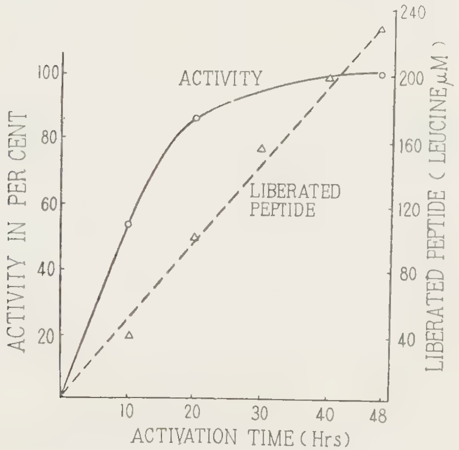

FIG 1. Activity and peptide liberation.

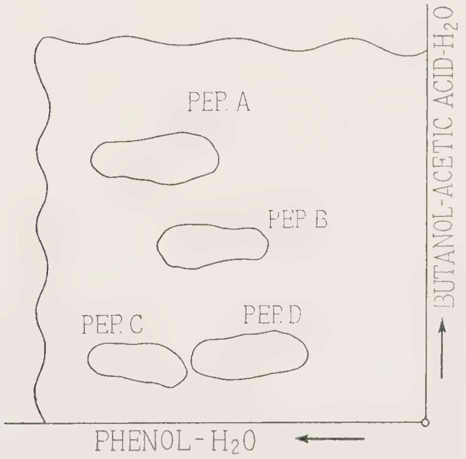

FIG. 2. Paper chromatogram of liberated peptides in the slow activation under dialysis.

Each of four spots which was detected with ninhydrin on two-dimensional paper chromatogram (as shown in Fig. 2.), was eluted with water and hydrolyzed in 6 N hydrochloric acid at 110° for 12 hours. The constituent amino acids of the four peptides were found chromatographically with the same solvents as above described to be as shown in Table I.

TABLE I

Constituent Amino Acid Residues of the Liberated Peptides

Peptide	Constituent amino acid residues
Pep. A	Leu,** Val, Ala, Gly, Glu, Thr, Cys, Asp, Ser, Lys
Pep. B§	Leu,** Val
Pep. C	Ala, Gly, Glu, Thr, Cys, Asp, Ser, Lys, Arg
Pep. D	Leu,** Val, Ala, Gly, Glu, Thr, Cys, Asp, Ser, Lys, Arg*

* It is rather vague on the chromatogram.

** It means either leucine, isoleucine or phenylalanine.

§ It is contaminated by small quantities of several other amino acids.

Slow Activation of α-Chymotrypsinogen—The slow activation of α-chymotrypsinogen was made without dialysis by the method descrbed in the previous work (*1*). Then, the degree of proteolysis was determined by measuring the increase of ninhydrin colour value of the activation mixture, and it was also expressed as per cent of the maximum increase (Fig. 3).

The activated mixture (42 ml.), which had previously contained 1000 mg. of α-chymotrypsinogen protein and 1.5 mg. of trypsin one, was acidified to pH 3.5 by several drops of 6 N hydrochloric acid and dialyzed at 5° for 15 hours against 200 ml. of 0.0025 N hydrochloric acid. The liberated peptides which were dialyzed into the hydrochloric acid sollution were treated as follows and converted to dinitrophenyl (DNP)-derivatives.

The hydrochloric acid solution was concentrated *in vacuo* to about 5 ml. and the precipitated salt was filtered and washed with 3 ml. of water. The filtrate was combined with the washing water, and the mixture was added with 500 mg. of sodium bicarbonate and 500 mg. of dinitrofluorobenzene(DNFB) dissolved in 10 ml. of alcohol, and shaken mechanically for 3 hours. Then the solution was desiccated *in vacuo* to dryness and dissolved in 5 ml. of water and shaken thrice with

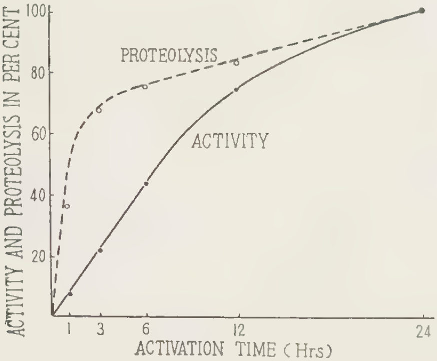

FIG. 3. Activity and proteolysis during slow activation.
(The concentration of the zymogen, 2.4%. Chymotrypsinogen: Trypsin, 670:1. Temperature, 5°.)

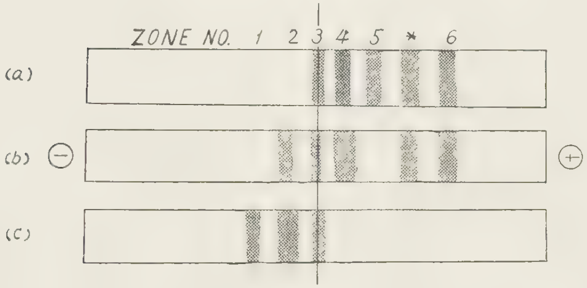

FIG. 4. Paper ionophoreses of DNP-peptides.

Inonophoreses were made on filterpaper No. 50 (4 cm × 30 cm, Toyo Filter Paper Co. Ltd.) with $N/10$ phosphate buffer (pH 6.1) (300 V, 10∼15 mA, 6 hours). (a), (b) and (c) show the ionophoretic patterns of DNP-peptides which obtained by extraction with ether, ethylacetate and methylethylketone, respectively.

* It was found to be decolorized by acidification with hydrochloric acid, and supposed to be dinitrophenol.

10 ml. of ether for removal of the excess of DNFB, and acidified with 6 N hydrochloric acid prior to successive extraction of the DNP-peptides with ether, ethylacetate and methylethylketone. Each of the extracted DNP-peptides was subjected to paper ionophoreses in dark to prevent the photodegradation of DNP-peptides, and the results were shown in Fig.

4. Paper ionophoreses were made on filterpaper No 50 (Toyo Filter Paper Co. Ltd., 4×30 cm) with *N*/10 phosphate buffer (pH 6.1) for 6 hours (300 V, 10∼15 mA).

As shown in Fig. 4 (c), the DNP-peptides which obtained by the extraction with methylethylketone was separated into two deep yellow zones (zone 1 and 2) and one faint yellow zone (zone 3). Two of them (zone 1 and 2) were cut, eluted by water, desiccated to dryness and subjected once more to the above procedure in order to isolate DNP-peptide. Each of the isolated DNP-peptides (DNP-peptide 1 and 2) was hydrolyzed in 6 *N* hydrochloric acid at 110° for 8 hours. DNP-amino acids in the hydrolysate were extracted by ether and characterized by chromatography on silicagel column according to the method of Sanger (*8*). At the same time, free amino acids in the hydrolysate were characterized by two-dimension paper chromatography with phenol-water (9:1) and *n*-butanol-acetic acid-water (4:1:1).

The results showed that DNP-peptide 1 was composed of one DNP-amino acid and one free amino acid, and identified to be DNP-seryl-arginine. DNP-Serine and an unidentified water soluble DNP-amino acid were found to exist in DNP-peptide 2, and several free amino acids, *i.e.* glutamic acid, aspartic acid, glycine (or serine), alanine and two or three other amino acids (or peptides) were also detected in the hydrolysate of DNP-peptide 2. Furthermore, DNP-peptide 2 was partly hydrolyzed in acetic acid-HCl (1:1) at 37° for 6 days (in sealed tube), and the resulted DNP-peptides were extracted by methylethylketone and isolated by ionophoreses. Only one DNP-peptide was detected in that case except the original DNP-peptide (DNP-peptide 2), and identified to be DNP-seryl-arginine by the same procedure as above mentioned.

The DNP-peptides which obtained by the extraction with ether were separated as shown in Fig. 4 (a) into two deep yellow zones (zone 4, and 6) and two faint yellow zones (zones 3 and 5). The deep ones (zones 4 and 6) were cut, eluted by water, desiccated to dryness and subjected once more to the above mentioned procedure. DNP-peptide 4 and 6 were respectively hydrolyzed and treated by the same procedure as previously mentioned. The results showed that DNP-peptide 6 was composed of one DNP-amino acid and one free amino acid and was identified to be DNP-threonyl-aspartic acid. DNP-threonine and an unidentified water soluble DNP-amino acid, and several free DNP-amino acids were found in the hydrolysate of DNP-peptide 4. DNP-peptides extracted by ethylacetate was separated into four faint yellow zones, and DNP-peptide 6 in Fig. 4 (b) was also isolated and identified to be DNP-threonyl-aspartic acid.

DISCUSSION

It has been already recognized by Kunitz and Northrop (*5*) that an independent proteolysis is accompanied by the tryptic conversion of chymotrypsinogen to chymotrypsin. They suggested that the conversion per se not involved the separation of nitrogenous products and that in such case an internal rearrangement would account for the formation of enzyme. This work was later confirmed by the more thorough study by Butler (*9*) and the study of the rapid activation by Jacobsen (*10*). Jacobsen postulated that an initial tryptic hydrolysis of one peptide bond yields an unstable intermediate, which was designated as π-chymotrypsin, and the chymotrypsin is transformed by further tryptic hydrolysis to δ-chymotrypsin and by chymotryptic hydrolysis to α-chymotrypsin. Further studies of the rapid activation by Neurath and his coworkers (*11*, *12*, *13*) and Desnuelle and his coworkers (*14*, *15*), established experimental proof for its details. They suggested that amino acid sequence involved in activation is leucyl-seryl-arginyl-isoleucyl-valine, and that arginyl-isoleucine bond is opend in the trypsin-catalyzed formation of π-chymotrypsin. The subsequent, chymotrypsin-catalyzed conversion of π-to δ-chymotrypsin was suggested to involve the hydrolysis of leucyl-serine bond, giving rise to a dipeptide and C-terminal leucine group.

However, Neurath and Bettelheim (*13*) indicated also that increasing the rate of activation does indeed depress the rate of the (presumably chymotryptic) splitting of a bond involving the amino group of alanine. As a possible explanation, they postulated that the splitting of this bond takes place more rapidly in chymotrypsinogen than in the chymotrypsins.

In the previous work (*1*), α-chymotrypsinogen was activated by the method of Kunitz and Northrop (*5*) so as to produce mainly α-chymotrypsin and studied electrophoretically, whereby the appearance of twelve components was recognized in the course of the activation for 24 hours. It was thus concluded by the author that the slow activation is not a simple process as previously supposed, but a highly complex one.

Furthermore, *N*-terminal amino acids of the activation mixture were determined by DNP-method (*3*), and the results indicated that three *N*-terminal groups, *i.e.* alanine, isoleucine (or leucine) and threonine were detected almost stoichiometrically in the activation mixture for 24 hours, whereas another three *N*-terminal groups, *i.e.* serine. glycine and aspartic acid did not amount to about 0.5 mole per mole α-chymotrypsinogen, and that in the initial stage of the activation, the splitting of a bond involving the amino-group of alanine was rather more predominat than that of arginyl-isoleucine bond (Table II).

TABLE II

N-Terminal Amino Acids of the Activation Mixture (3)

	N-Terminal amino acids* in moles per mole α-chymotrypsinogen				
Activation time *hours*	0	3	6	12	24
Aspartic rcid	0.05	0.25	0.31	0.28	0.27
Serine	0.04	0.47	0.48	0.49	0.49
Threonine		0.47	0.50	0.56	0.72
Glycine	0.04	0.36	0.37	0.40	0.43
Alanine		0.65	0.65	0.70	0.71
Isoleucine**		0.32	0.52	0.71	0.78
Activity per cent	0	33	46	64	100

* Not corrected for the decomposition of DNP-amino acids during acid hydrolysis, and molecular weight of α-chymotrypsinogen is assumed to be 25,000 (*16*).

** It has been known that DNP-isoleucine is present in the hydrolysate of chymotrypsin partly as DNP-isoleucylvaline, which is very resistant to hydrolysis (*17*). This DNP-peptide ran as DNP-phenylalanine in the chromatographic system used in this experiment, and the figures obtained were added to those for free DNP-isoleucine.

The present experimental results shown in Fig. 3, indicate that in the slow activation of α-chymotrypsinogen, the trypsin-catalyzed formation of π-chymotrypsin is a rather slow process, and the small quantities of the chymotrypsin may catalyze rapidly the conversion of α-chymotrypsinogen to an intermediate protein without proteolytic activity, which was converted subsequently to α-chymotrypsin by tryptic or chymotryptic hydrolysit or both.

In the present experiment, the slow activation took place under dialysis, and the quantities of the liberated peptides which passed through a cellophane bag, increase almost lineally during the whole activation course (Fig. 1). Although all the liberated peptides do not instantly pass the cellophane membrane, it seems likely that the initial rapid proteolysis* in Fig. 3 depends mainly upon the splitting of several peptide bonds including the amino group of threonine or alanine, which are responsible to neither enzymatic activity nor liberation of peptides.

As shown in Fig. 4, the slow activation of α-chymotrypsinogen liberates several peptides, two of which were isolated and identified to be threonyl-aspertic acid and seryl-arginine, and another one of which gives seryl-arginine as its degradation product by partial hydrolysis (acetic acid-hydrochloric acid=1 :1, 37°,6 days). The result obtained in the case of slow activation under dialysis, has also shown the liberation of four peptides, amino acid constitution of which might suggest that the slow activation is accompanied with the liberation of a large peptide as follows.

[Leu, Val, Ala, Glu, Gly, Thr, Asp, Cys, Lys, Ser, Arg]

As the slow activation without dialysis may give rise to further non-specific hydrolysis of the large peptide, it is probable that the whole slow activation course should liberate the dipeptides like thereonyl-aspartic acid and seryl-arginine.

SUMMARY

1. α-Chymotrypsinogen was activated under dialysis, and the correlation between enzymatic activity and peptide liberation was investigated.

2. In the slow activation, the initial rapid proteolysis was found to be responsible to neither enzymatic activity nor liberation of peptides.

3. The slow activation of α-chymotrypsinogen is accompanied by the formation of several peptides, two of which were identified to be seryl-arginine and threonyl-aspartic acid, and which probably arise from one or two common precursor composed of several amino acid residues.

The author wishes to thank professor Shiro Akabori for his guidance through-

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* It has been reported by Desnuelle *et al.* (15).

out this work, and also Mr. Yoshimi Okada and Miss Hisako Yamada for their valuable technical assistances.

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The Journal of Biochemistry, Vol. 43, No. 4, 1956

CHROMATOGRAPHY OF CYTOCHROME c ON ION EXCHANGE RESIN

Sirs:

In a recent paper dealing with the purification and the properties of yeast cytochrome c (*1*), one of us reported that this pigment seems to be similar to mammalian cytochrome c in chromatographic behaviours on Amberlite XE-64, rather than to those from *Ustilago* or *Rhodospirillum*. Ishimoto (*2*) also reported the same similarity between cytochrome c_3 from *Desulfovibrio desulfricans* and mammalian cytochrome c in their displacement chromatography according to the method of Margoliash (*3*).

These "similarities," however, do not necessarily imply that these pigments cannot be distinguished from each other by the ion exchange resin chromatography. As shown in Fig. 1, the difference of R values

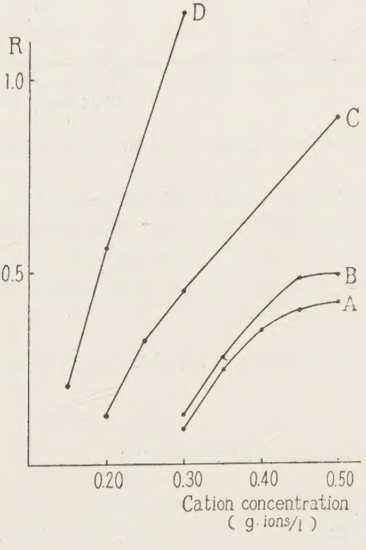

FIG. 1. Effect of cation concentration on R-values of cytochromes. A: yeast cytochrome c (oxidized), B: yeast cytochrome c (reduced), C: horse heart cytochrome c (oxidized), D: cytochrome c_3.

among these cytochromes on Amberlite XE-64 (200–400 mesh) column equilibrated with phosphate buffer (pH 7.0) of various cation concentration was rather distinct. Based upon these chromatographic difference, the synthetic mixtures of these pigments were easily separated by the elution analysis according to the method of Boardman and Partridge (*4*). Fig. 2 shows a representative result of the elution chromatography.

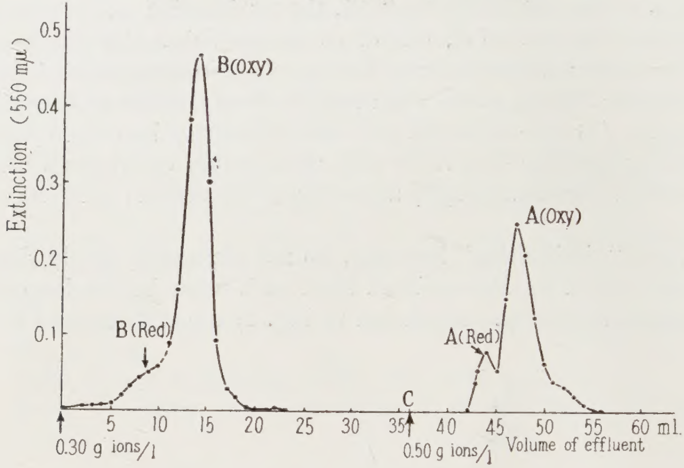

FIG. 2. Separation of yeast (A) and horse heart (B) cytochrome c.

Column of Amberlite XE-64 (200–400 mesh) 0.9 × 10 cm. Buffer: Sodium phosphate (pH 7.0). Na^+ concentration was changed from 0.30 to 0.50 g. ions/l at the point C. Temperature: 18°. Cytochrome c in each fraction was estimated photometrically at 550 mμ after reduction.

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